Dissecting the role of p53-mediated metabolic regulation in tumor suppression

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ABSTRACT

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The p53 tumor suppressor protein has been well-characterized for its role in inducing growth arrest, senescence, and apoptosis upon various types of stresses. Recently, however, roles of p53 have expanded beyond the canonical functions, and now include cellular processes such as metabolism, oxidative balance, and ferroptosis. Through RNA-seq screening, we first identified phosphoglycerate dehydrogenase (PHGDH), a rate-limiting enzyme in the serine biosynthesis pathway, as a novel metabolic target of p53. p53 suppresses PHGDH expression and inhibits de novo serine biosynthesis. Notably, upon serine starvation, p53-mediated cell death is significantly enhanced in response to Nutlin-3 treatment. Moreover, *PHGDH* has been demonstrated to be frequently amplified in human melanomas. We found that PHGDH overexpression significantly suppresses the apoptotic response, whereas RNAi-mediated knock-down of endogenous PHGDH promotes apoptosis under the same treatment. Together, our findings demonstrate an important role of p53 in regulating serine biosynthesis through suppressing PHGDH expression, and reveal serine deprivation as a novel approach to sensitize p53-mediated apoptotic responses in human melanoma cells.

In addition, we also identified spermidine/spermine N^1-acetyltransferase 1 (SAT1) as a novel metabolic target of p53. SAT1 is a rate-limiting enzyme in polyamine catabolism critically involved in the conversion of spermidine and spermine back to putrescine. Surprisingly, we found that activation of SAT1 expression induces lipid peroxidation and sensitizes cells to undergo ferroptosis upon reactive oxygen species (ROS)-induced stress, which also leads to suppression of tumor growth in xenograft tumor models. Notably, *SAT1* expression is down-regulated in human tumors, and CRISPR-cas9-mediated knockout of *SAT1* partially abrogates p53-mediated ferroptosis. Moreover, SAT1 induction is correlated with the expression levels of arachidonate 15-lipoxygenase (ALOX15), and SAT1-induced ferroptosis is significantly abrogated in the presence of PD146176, a specific inhibitor of ALOX15. Together, these data indicate a novel regulatory role of p53 in polyamine metabolism and provide insight into the regulation of p53-mediated ferroptotic responses.

Our studies on PHGDH and SAT1 led us to the question of whether these unconventional functions of p53 contribute to its role as a tumor suppressor. In fact, previous view regarding the mechanism of p53-mediated tumor suppression, which was long thought to be growth arrest, apoptosis, and senescence, has recently been challenged by several knockout and knock-in mouse studies. Previously, we established mice ($p53^{3KR/3KR}$) in which p53 acetylation at lysine residues K117, K161, and K162 were abolished by replacing lysine with arginine. $p53^{3KR/3KR}$

mice completely lost p53-mediated cell cycle arrest, apoptosis, and senescence functions in response to stresses. However, unlike p53-null mice which rapidly develop spontaneous thymic lymphomas, all of the *p53*$^{3KR/3KR}$ mice remain tumor-free, indicating that other aspects of p53 functions are sufficient to prevent tumor formation. Notably, p53^{3KR} retains the ability to regulate metabolic targets including TIGAR and SAT1, as well as ferroptosis regulator SLC7A11. In this study, we have identified two novel acetylation sites- K98 and K136, in the mouse p53 DNA-binding domain. Whereas loss of K98 or K136 acetylation (p53^{K98R}, p53^{K136R}) alone has modest effect on p53 transcriptional activity, simultaneous mutations at all of these acetylation sites (p53^{4KR98}: K98R+3KR, p53^{4KR136}: K136R+3KR, p53^{5KR}: K98R+K136R+3KR) completely abolish the ability of p53 to regulate TIGAR, SAT1, and SLC7A11. In addition, p53^{4KR98}, p53^{4KR136}, and p53^{5KR} are defective in Erastin-induced ferroptosis. Notably, *p53*$^{4KR98/4KR98}$, *p53*$^{4KR136/4KR136}$, and *p53*$^{5KR/5KR}$ knock-in mice lost intact tumor suppression and developed spontaneous tumors. This suggests that p53-mediated ferroptosis may function as a critical barrier to prevent tumor formation independently from growth arrest, apoptosis, and senescence. Interestingly, both *p53*$^{4KR98/4KR98}$ and *p53*$^{4KR136/4KR136}$ mice displayed significantly delayed tumorigenesis comparing with p53-null and *p53*$^{5KR/5KR}$ mice. We found that unlike p53^{5KR}, p53^{4KR98} retains the capacity to inhibit mammalian target of rapamycin (mTOR) signaling pathway through activating the expression of two mTOR negative regulators, Sestrin2 and

DDIT4. Altogether, our findings underscore the extensive scope of p53 functions in metabolic regulation, oxidative stress response, and ferroptosis, and provide novel insights into the tumor suppression mechanism of p53.

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CHAPTER 1

Introduction

1.1 p53 tumor suppressor

The p53 protein is widely accepted as “cellular gatekeeper” or “the guardian of the genome” because of its pivotal role in suppressing tumorigenesis [1, 2]. Mutations that perturb p53 function have been found in more than 50% of human cancers [3-6]. Germline mutation of p53, which results in only one functional allele of *TP53* gene, is associated with Li-Fraumeni syndrome that confers high familiar risk of cancer [7, 8]. In addition, mice deficient in *Trp53* (gene that encodes mouse p53) are prone to develop early onset spontaneous tumors [9].

The human p53 protein consists of an amino (N-) terminal transactivation domain (residue 1-62), a proline rich domain (residue 63-97), a central DNA binding domain (residue 100-300), a tetramerization domain (residue 307-355), and carboxyl (C-) terminal regulatory domain (residue 356-393) [10, 11]. The transactivation domain is responsible for recruiting transcriptional machinery and co-activators for transcription activation. The central DNA binding domain of p53 binds to specific DNA sequences termed “p53 responsive element” or “p53 consensus binding site”, which contains two decamers 5’-PuPuPuC(A/T)(T/A)GPyPyPy-3’ (Pu: purine; Py: pyrimidine) separated by 0-13 base pairs [12, 13]. The tetramerization domain enables p53 subunits to form tetramer, and the C-terminal regulatory domain modulates p53 transcription activity through abundance of post-translational modifications [14].

p53 mainly functions as a transcription factor to coordinate cells to respond to a variety of cellular stresses, and monitor signaling pathways involved in cell cycle arrest, apoptosis, senescence, cellular metabolism, autophagy etc. Under physiological condition, p53 is kept at an extremely low level through a negative-feedback loop with Mouse Double Minute 2 (MDM2, human protein is often called HDM2). MDM2 is a direct p53 transcriptional target which gets up-regulated upon p53 activation. It is also a p53-specific E3 ubiquitin ligase that can promote the ubiquitination and subsequent proteasomal degradation of p53. Upon cellular stresses such as DNA damage, ionizing radiation, hypoxia and oncogenic stress, p53 gets rapidly stabilized and activated [2, 15]. Classical models for p53 activation consist of p53 stabilization by ATM/ATR-mediated phosphorylation, sequence-specific DNA binding, co-activator/co-repressor recruitment, and subsequent transcription activation or repression of downstream target genes [16]. As a result, p53 coordinates cells to respond to various stresses, protects them from genomic instability, and ultimately, prevents tumor formation.

1.2 Post-translational modifications of p53

p53 is a very dynamic protein which requires a fine-tuning regulation mechanism that is mediated by an abundance of post-translational modifications (Figure 1.1). Post-translational modifications play a pivotal role in controlling p53 stability, activity, and more recently described, differential regulation of downstream targets. Under unstressed condition, p53 is

maintained at low levels through ubiquitination and subsequent proteasomal degradation [17]. When cells encounter stress, the ubiquitination of p53 is inhibited, and p53 is rapidly stabilized and activated in the nucleus mainly through phosphorylation and acetylation. This also enables p53 to form homotetrameric complex, which acts to bind to sequence-specific promoters of its downstream target genes, and recruit transcriptional machineries as well as co-activators for transcription activation [18]. In addition, recent findings revealed that p53 could also undergo methylation, neddylation and sumoylation, all of which result in specific p53 responses [19-21].

Figure 1.1

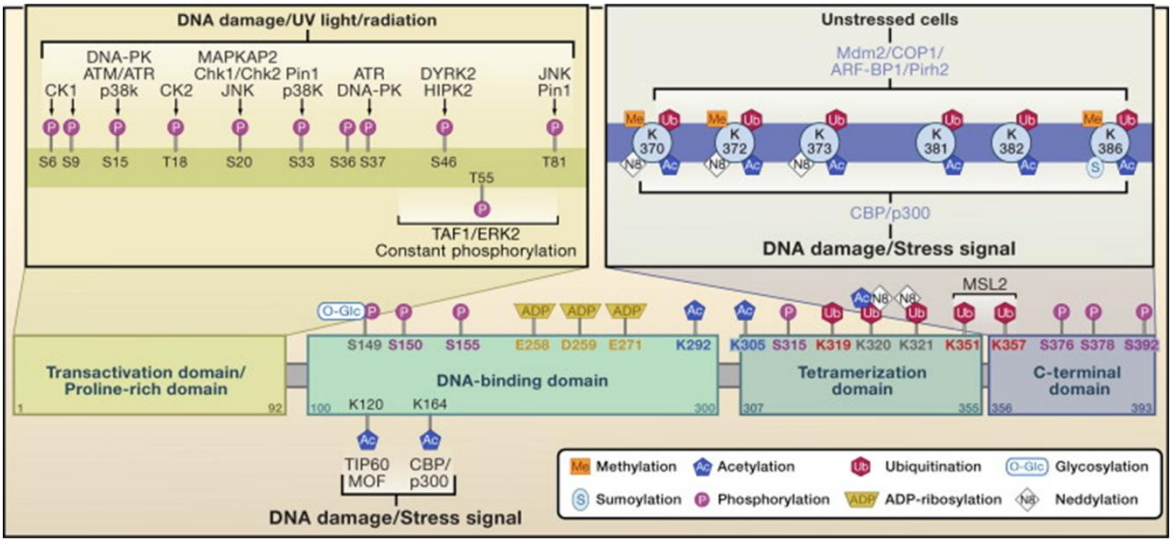

Figure 1.1 Overview of p53 post-translational modifications. The p53 protein is reported to be modified on more than 36 amino acids by indicated modifying enzymes which include ubiquitination, phosphorylation, acetylation, methylation, neddylation, and sumoylation. (Figure cited from Kruse et al., *Cell* 2009)

1.2.1 Ubiquitination

Under physiological condition, p53 protein level is tightly controlled by ubiquitin-mediated proteasomal degradation. Mdm2 is a major p53 E3-ubiquitin ligase and negative regulator of p53 [22]. Mdm2 mainly targets six p53 lysine residues in the C-terminal domain for poly-ubiquitination and subsequent proteasomal degradation [23]; it also directly binds to p53 transactivation domain and inhibits the transcriptional activity of p53 through blocking the recruitment of basic transcriptional machinery and coactivator [24]. Interestingly, p53 still undergoes degradation in the cells of Mdm2-null mice, suggesting the existence of Mdm2-independent degradation pathways. In fact, p53 can also be poly-ubiquitinated by several other E3-ligases, such as COP1, Pirh2, and Arf-BP1, which contribute to the overall control of p53 levels in the cell [25-27]. Besides proteasomal degradation, ubiquitination of p53 also plays a role in regulating the cellular localization of p53. p53 is found to be mono-ubiquitinated by Mdm2 and other E3-ligases, and undergo nuclear export and translocation to cytoplasm, which is thought to play important roles in regulating apoptosis and autophagy through transcription-independent mechanisms [28-30].

1.2.2 Phosphorylation

Phosphorylation on serine (S) or threonine (T) residues mainly in p53 N-terminal transactivation domain is important for the activation of p53. S15 and S20 (S18 and S23 in

mouse p53) are two most extensively studied phosphorylation sites in p53, which are catalyzed by ATM/ATR/DNA-PK and Chk1/Chk2 kinases respectively [31, 32]. Phosphorylation of p53 is thought to disrupt the interaction between p53 and Mdm2, and result in p53 stabilization, recruitment of transcriptional machinery, and subsequent p53 activation. Interestingly, mice bearing single S18 or S23 to alanine (A) mutation in p53 exhibit only modest effect on p53 stability and transcriptional activity, indicating that these two phosphorylation sites have functional redundancy [33, 34]. However, S18/23A double-mutant p53 displayed greater defect in transcriptional activity, suggesting a synergetic role of these two phosphorylation sites in p53 activation [34].

1.2.3 Acetylation

Acetylation is a type of post-translational modification specifically occurs on lysine residues that was initially discovered in histones, and is known to have a major impact on transcriptional regulation [35]. p53 was later on found to be the first non-histone protein to be functionally regulated by acetylation and deacetylation [36, 37]. In response to stress, p53 is acetylated by histone acetyltransferase CBP/p300, and acetylation of p53 is known to promote the recruitment of cofactors and enhance target gene transcription [38]. Other p53 acetyltransferases such as Tip60 and hMOF also play an important role in regulating p53 transcriptional activity [39, 40]. p53 was first discovered to be acetylated at six lysine residues in

the C-terminal domain by CBP/p300 [36]. Interestingly, the C-terminal acetylation-deficient $p53^{6KR}$ knock-in mice showed impaired p53-dependent gene expression only in embryonic stem cells and thymocytes, but not in embryonic fibroblasts [41]. Similarly, another knock-in study in $p53^{7KR}$ mice also demonstrated that C-terminal acetylation did not affect p53-mediated cell cycle control and apoptosis in embryonic fibroblasts [42]. These findings suggested that the regulatory effects of p53 C-terminal acetylation may be cell type-specific, but does not seem to have a major impact on cell cycle or apoptosis control. Notably, recent studies show that p53 acetylation is not limited to C-terminal. Both studies have shown that p53 is acetylated at lysine K120 located in central DNA-binding domain by hMOF and Tip60, two acetyltransferases that belong to the MYST family [39, 40]. The K120 lysine residue is conserved among all species that have functional p53 genes, suggesting that this acetylation may possess an evolutionarily conserved role [16]. It has been found that K120 acetylation occurs after DNA damage or oncogenic stress, and is required for p53 to activate proapoptotic target genes *Puma* and *Bax* [39, 43]. When K120 acetylation is abrogated by mutating lysine to arginine, p53-mediated Puma and Bax activation are impaired, while the expression of Mdm2 and p21 are not affected [39]. Furthermore, loss of K120 acetylation does not affect p53 stability or DNA-binding either [39]. K164 is another p53 acetylation site in the DNA-binding domain and it is acetylated by CBP/p300 [44]. Interestingly, loss of acetylation individually at K120, K164 or 6KR does not impair p53's ability to activate

p21, suggesting that these acetylation sites may compensate each other through functional redundancy. However, simultaneous loss of acetylation at K120, K164, and six C-terminal lysine residues ($p53^{8KR}$) completely abolishes p53's ability to induce p21 expression and growth arrest, while retains the ability of p53 to bind DNA and induce Mdm2-p53 negative feedback loop [44]. These results also suggested that p53 acetylation is important for the regulation of promoter specific activation, although the molecular mechanism remains to be elucidated. Notably, p53 can also be deacetylated by distinct deacetylases including HDAC1 and Sir2α/Sirt1, and deacetylation of p53 is demonstrated to repress p53-mediated transcriptional activation, growth arrest, and apoptosis in response to DNA damage and oxidative stress [37, 45, 46]. Overall, acetylation and deacetylation of p53 provides a dynamic fine-tuning mechanism that allows p53 to activate target gene transcription in response to cellular stresses, and return to steady-state level after completion of DNA repair.

1.2.4 Methylation, Neddylation and Sumoylation

Beside ubiquitination and acetylation, lysine residues on p53 can also undergo methylation, neddylation, and sumoylation, and these modifications are thought to contribute to p53 promoter specificity. Monomethylation of p53 at lysine K372 by methyltransferase Set7/9 has shown to promote p53 activity [19]. In contrast, K370 and K382 methylation by Smyd2 and Set8/PR-Set represses p53 activity [47, 48].

Neddylation and Sumoylation are two ubiquitination-like modifications that involve in the addition of Neural precursor cell Expressed Developmentally Down-regulated protein 8 (Nedd8) and Small Ubiquitin-like Modifier (SUMO) molecules to the C-terminal lysine residues in p53. The exact roles of neddylation and sumoylation are not well defined. Some studies have reported that Mdm2-mediated neddylation on K370/372/373 and FBXO11-mediated neddylation on K320/321 inhibit p53 activity [20, 49]. In addition, one study demonstrated that sumoylation of p53 on K386 promotes the transcriptional activity of p53, while another group suggested that this modification induces cytoplasmic localization of p53 [21, 50].

1.3 Classical functions of p53: growth arrest, apoptosis and senescence

Growth arrest, apoptosis, and senescence are the most well characterized downstream events in response to the p53 activation, and are thought to be the major mediators of the tumor suppressive function of p53.

Growth arrest function of p53 is predominantly mediated by its downstream target gene *p21 (WAF1)*. p21 binds to a number of cyclin-Cdk complexes, inhibits their kinase activity, and therefore, blocks cell cycle progression at G1 and S phases [51]. Interestingly, $p21^{-/-}$ mice develop normally, and mouse embryonic fibroblasts (MEFs) derived from mice lacking *p21* are partially deficient in inducing G1 arrest upon DNA damage, suggesting the existence of p21-indepdendent pathway that contributes to p53-mediated cell cycle arrest [52]. GADD45 and

14-3-3σ are later characterized to be another two p53 downstream targets that play an important role in G2 arrest through inhibition of cyclinB-cdc2 complex [53, 54].

In response to cellular stresses, p53 could induce apoptosis through both transcription dependent and independent pathways. p53 activates the transcription of pro-apoptotic Bcl-2 family members including p53-upregulated modulator of apoptosis (PUMA), Bcl-2-associated X protein (BAX) and phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) [55]. PUMA and NOXA bind to mitochondrial anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-X_L, and Mcl-1, thus inhibit their interaction with Bax and lead to caspase activation and cell death [56, 57]. In addition, p53 could directly translocate to mitochondria and trigger apoptosis through activation of Bcl-2 family members [58].

Senescence is an irreversible cell-cycle arrest in response to either telomere shortening during normal aging, or persistent oncogenic signaling that can be induced by p53 [59, 60]. It is featured by its distinctive morphology change including flattened/enlarged shape, increased adherence, decreased replicative capacity, and increased expression of senescence-associated β-galactosidase (SA-β-GAL) [61]. The key effectors of p53-mediated senescence include p21, plasminogen activator inhibitor-1 (PAI-1), and E2F7 [62-65].

1.4. Non-canonical functions of p53

Recently, the functions of p53 have been largely expanded beyond the classical functions of cell cycle arrest, apoptosis and senescence, and now include metabolism, reactive oxygen species (ROS) regulation, autophagy, and ferroptosis (an iron-dependent oxidative cell death) (Figure 1.2).

Figure 1.2

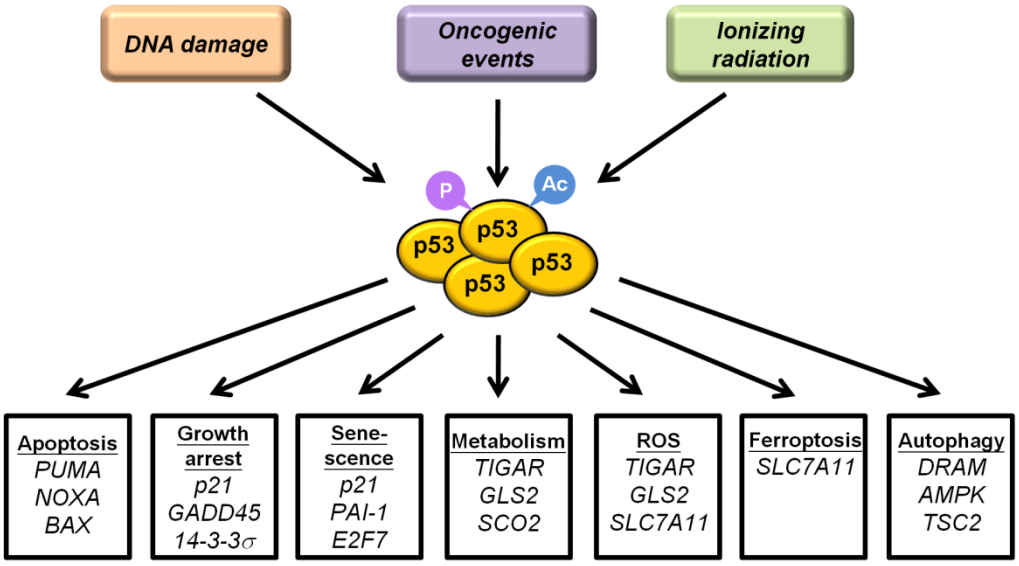

Figure 1.2. p53 regulates a wide variety of cellular functions. Upon cellular stresses such as DNA damage, oncogenic events, or ionizing radiation, p53 is stabilized and activated to induce a plethora of downstream cellular events through transcriptional regulation of corresponding target genes.

1.4.1 Metabolism and ROS regulation

Metabolic alteration of tumor cells has been recognized as a hallmark of cancer. In 1926, Otto Warburg first described the phenomenon that unlike normal cells which depend on mitochondrial respiration to produce energy, majority of cancer cells use glycolysis instead, and this phenomenon is named “the Warburg effect” [66]. Although aerobic glycolysis is much less efficient in ATP production compared with oxidative phosphorylation, it provides a much larger number of intermediates for the de novo macromolecule biosynthesis of protein, lipids, and nucleic acids. On the other hand, cancer cells also have a much higher rate of glucose uptake to meet their energy needs [67]. More recently, other metabolic alterations in glutaminolysis, amino acid metabolism, lipid metabolism as well as other biosynthetic and bioenergetic pathways have also been found in cancer, and all of these metabolic reprogramming are thought to be required for anabolic growth of cancer cells [68].

Studies in the past decade have demonstrated that p53 can counteract the effects of metabolic alteration through regulating numerous metabolic targets, resulting in inhibition of aerobic glycolysis, up-regulation of mitochondrial oxidative phosphorylation, and promotion of fatty acid oxidation [69]. In addition, several metabolic targets also function to increase antioxidant defense and protect cells from ROS-induced DNA damage and malignant transformation. TP-p53 induced glycolysis and apoptosis regulator (TIGAR), an enzyme that

converts fructose-2,6-bisphosphate to fructose-6-phosphate, was identified to be a direct target of p53 [70]. This conversion shunts the glycolytic intermediates into pentose phosphate pathway for NADPH production and glutathione recycling, therefore reducing glycolytic rate and decreasing intracellular ROS [70]. Additionally, p53 inhibits glycolysis through transcriptionally repressing the expression of glucose transporters such as GLUT1, GLUT3, and GLUT4 [71]. Besides glycolysis, p53 also plays an important role in the regulation of mitochondria and oxidative phosphorylation. p53 has been indicated to induce the synthesis of cytochrome c oxidase 2 (SCO2), a key regulator of the cytochrome C oxidase complex in the electron transport chain, which functions to increase mitochondrial respiration [72]. Glutaminase 2 (GLS2) is also a p53 target that converts glutamine to glutamate, and functions to enhance TCA cycle and mitochondrial respiration through promoting the production of glutamate and α-ketoglutarate. [73, 74]. Additionally, expression of GLS2 protects the cells from oxidative stress-induce cell death by increasing the synthesis of reduced glutathione (GSH) and decreasing ROS levels [73, 74].

Recent findings have also linked p53 to the regulation of lipid metabolism. Fatty acids (FAs) is a major group of lipids that constitute the cell membrane, and the regulation of FA metabolism is critical for maintaining energy balance [75]. In fact, to support anabolic growth, many cancer cells exhibit increased FA synthesis and decreased FA oxidation (FAO), both of which could be

counteracted by p53 through the regulation of several metabolic target genes. Upon glucose deprivation, activated p53 induces the expression of guanidinoacetate *N*-methltransferase (GAMT), which leads to increased FAO in both liver tissue and cultured cells [76]. Another p53 target lipin1 (LPIN1), when induced upon nutrient starvation, could cooperate with peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) and peroxisome proliferator-activated receptor alpha (PPARα) to enhance FAO and inhibit FA synthesis through regulating the expression of genes involved in FA metabolism pathways [77, 78]. NAPDH, a cofactor that is produced from pentose phosphate pathway, is required for lipid synthesis. In fact, p53 has been indicated to inhibit the activity of glucose-6-phophate dehydrogenase (G6PD), a rate-limiting enzyme in the pentose phosphate pathway, and therefore, reduce NAPDH levels and lipid synthesis [79]. Furthermore, p53 inhibits lipogenesis through repressing the expression of sterol regulatory element-binding protein SREBP1c, a transcription factor that regulates lipogenesis [80].

1.4.2 Ferroptosis

Our recent study has revealed that p53 can induce ferroptosis by suppressing the expression of SLC7A11, a component of the cystine/glutamate antiporter (system x_c^-) [81]. Ferroptosis is form of non-apoptotic, iron-dependent and programmed cell death that is known to be driven by membrane lipid peroxidation. Ferroptosis is morphologically distinct from apoptosis and

necroptosis, which is featured by the presence of shrunken mitochondria, increased mitochondria membrane density, and the absence of DNA fragmentation [81]. Erastin and RSL3 are the first two ferroptosis-inducing compounds that were discovered using small-molecule high-throughput screening [82, 83]. Cells treated with Erastin or RSL3 died in the absence of apoptosis markers [83]. Knockdown or pharmacological inhibition of RIPK1, a key component of necroptosis, did not rescue Erastin-induced cell death [84]. Moreover, iron chelators were found to inhibit RSL3-induced cell death, suggesting that cellular iron is required for this type of cell death [83]. Glutathione peroxidase 4 (GPX4) is a target protein for RSL3 that was recently identified to be a central regulator of ferroptosis. Using glutathione as a cofactor, GPX4 decreases cellular ROS levels and reduces lipid hydroperoxides on cellular membranes [85]. Knockout of *Gpx4* led to accumulation of lipid ROS in mouse brain tissue and caused rapid motor neuron degeneration by ferroptosis [86, 87]. Conditional ablation of *Gpx4* in mouse T cells also resulted in T cell ferroptosis and decreased immune response [88]. This suggests that ferroptosis plays an important role in development. System x_c^- is another key regulator of ferroptosis, and it can by inhibited by Erastin. System x_c^- is responsible for the uptake of cystine, which is a precursor for glutathione synthesis. Inhibition of System x_c^- by Erastin depletes the intracellular cystine levels, and results in glutathione depletion and ferroptosis [84]. Our recently study revealed that through repressing the expression of SLC7A11, a component of System x_c^-, p53inhibits cystine take

and sensitizes cells to ferroptosis upon ROS-induced stress [81]. Moreover, overexpression of SLC7A11 significantly abrogated the tumor growth suppression activity of $p53^{3KR}$, indicating that p53-mediated ferroptosis is important for its tumor suppression functions [81].

1.4.3 Autophagy

Autophagy is an intracellular degradation system that delivers long-lived proteins and damaged organelles for degradation and recycling in lysosomes. It functions to eliminate unwanted cellular components and protect cells from nutrient starvation. p53 has been revealed to both induce and inhibit autophagy, depending on its sub-cellular localization [89]. Nuclear p53 promotes autophagy through transcriptional activation of several target genes that stimulate autophagy. DRAM (damage-regulated autophagy modulator) is a direct target of p53 that functions as a lysosomal membrane protein to induce autophagy [90]. Although overexpression of DRAM alone induces minimal cell death, it is found to be essential for p53-mediated apoptosis [90]. Moreover, the expression of DRAM is frequently decreased in primary tumors accompanied by retention of wild-type p53, suggesting that DRAM-mediated autophagy may contribute to p53-mediated tumor suppression [90]. mTOR (mammalian target of rapamycin) is a sensor for cellular nutrients and energy levels, and inhibits autophagy as one of its downstream events . AMP-activated protein kinase (AMPK) and tuberous sclerosis complex (TSC1/2) are negative regulators of mTOR, when activated upon energy deprivation, would promote

autophagy through inhibition of mTOR [91]. In fact, p53 can transcriptionally activate both the β1 and β2 subunits of AMPK as well as TSC2, resulting in induction of autophagy [92]. In contrast to nuclear p53, cytoplasmic p53 seems to inhibit autophagy through transcription-independent mechanisms, although the exact molecular mechanism remains to be elucidated. Some studies have demonstrated that the inhibitory effect of autophagy is determined by the ratio of cytoplasmic p53 to nuclear p53 levels, with higher cytoplasmic p53 resulting in greater autophagic inhibition [28, 93].

1.5 Mechanism of p53-mediated tumor suppression

Since p53 functions as a tumor suppressor through regulating a wide variety of target genes involved in different functional areas, it is important to understand which functions of p53 contribute to tumor suppression. The growth arrest, apoptosis, and senescence functions of p53 have long been accepted as the major mechanism to suppress tumorigenesis. However, several recent studies have challenged this old view, indicating that the tumor suppressive function of p53 can be achieved in the absence of cell cycle arrest, apoptosis, and senescence.

1.5.1 Growth arrest, apoptosis, and senescence are dispensable for p53-mediated tumor suppression

p21 single knock-out and *Puma/Noxa* double knock-out mice were first generated to evaluate the role of growth arrest and apoptosis in tumor suppression. However, neither of them

developed spontaneous tumor formation, possibly due to the compensation for each other between these two functions [52, 94, 95]. Surprisingly, later study also failed to observe any increased tendency of tumorigenesis in triple knock-out mice of *p21*, *Puma*, and *Noxa* [96]. Notably, mouse $p53^{3KR}$, an acetylation deficient mutant of p53 on lysine residues K117, K161, and K162 (homologous to K120 and K164 in human p53), is defective in p53-dependent cell cycle arrest, senescence, and apoptotic responses [97]. However, unlike p53-null mice which develop early onset of thymic lymphomas within 6 months of age, all of the $p53^{3KR/3KR}$ knock-in mice remain tumor free and healthy up to 16 months [97]. Similarly, another p53 knock-in mouse model containing two mutated residues in codon 25 and 26 at N-terminal transactivation domain ($p53^{25,26/25,26}$) also showed intact tumor suppression although $p53^{25,26}$ is compromised in its ability to activate the transcription of apoptotic and growth arrest targets [98]. These studies suggest that the growth arrest, apoptosis, and senescence functions of p53 are dispensable for it to suppress tumor formation, underscoring the potential significance of non-canonical functions of p53 in contributing to p53-mediated tumor suppression. Interestingly, $p53^{3KR/3KR}$ mice retain the ability to regulate metabolism and induce ferroptosis through certain target genes, including *TIGAR*, *GLS2*, *GLUT1/4*, and *SLC7A11*, and these functions of p53 may play an important role in p53-mediated tumor suppression [81, 97].

1.5.2 Role of non-canonical functions of p53 in tumor suppression

Although p53 can regulate a variety of metabolic targets that function to counteract the metabolic alterations commonly observed in cancer cells, such as increased glycolysis, decreased mitochondria respiration and lipid oxidation, the link between metabolic reprogramming and tumorigenesis remains elusive. Studies have shown that p53 metabolic targets have both tumor suppressive and tumor promoting properties. Loss of GLS2, a p53 metabolic target in glutamine metabolism and ROS regulation, has been indicated to correlate with neoplastic transformation in human hepatocellular carcinomas [99]. In addition, low expression of SCO2, a p53-regulated target in mitochondria respiration, has been revealed to be associated with poor prognosis in human invasive breast cancers [100]. These results suggest that p53-mediated regulation of GLS2 and SCO2 may contribute to tumor suppression possibly by prevent metabolic transformation. In contrast, TIGAR, a bona fide p53 metabolic target that functions to inhibit glycolysis and increase antioxidant production, is demonstrated to have tumor-protective activities. Cheung et al. reported that *TIGAR* knockout mice displayed decreased tumor burden compared with *TIGAR* wild-type mice in an intestinal adenoma mouse model [101]. They revealed that Tigar expression increases GSH: GSSG ratio in intestinal tumor cells, thus preventing the accumulation of ROS [102]. Additionally, Tigar also up-regulates the synthesis of nucleotide and allow cancer cells to proliferate more efficiently [102]. In fact, the antioxidant

function of p53 seems to play a dual role in tumor formation and development. While decreasing the intracellular ROS level by p53 could protect normal cells from oxidative damage to DNA, and potentially prevent malignant transformation; it also could support the survival and growth of cancer cells by alleviating the toxicity from oxidative stresses. Further studies are required to dissect the role of p53-mediated metabolic regulation and antioxidant function in tumor initiation and progression.

Interestingly, recent discovery of the link between p53 and ferroptosis has shed light on the previously unanticipated tumor suppression mechanism of p53. Besides modulating ROS levels, p53 can also directly respond to ROS stress or accumulation through repressing the expression of SLC7A11, and result in inhibition of cystine uptake, increase of lipid peroxidation, and induction of ferroptosis. In fact, SLC7A11 is found to be overexpressed in many types of human cancers [81]. Jiang et al. also revealed that $p53^{3KR}$, an acetylation-deficient p53 mutant that is defective in p53-mediated growth arrest, apoptosis, and senescence, retains the ability to repress SLC7A11 expression and trigger ferroptosis in response to oxidative stress [81]. Although $p53^{3KR}$ retains intact tumor suppression activity, reconstitution of robust SLC7A11 expression in $p53^{3KR}$ cancer cells significantly abrogated its ability to suppress tumor formation in xenograft mouse models [81]. Moreover, a ferroptosis inhibitor (Ferrastatin-1) was demonstrated to reverse the developmental defect of $p53^{3KR/3KR}Mdm2^{-/-}$ embryos, suggesting that p53-mediated ferroptosis

also contributes to embryonic development and the lethality associated with loss of *Mdm2* [81]. Additionally, a recent study reported that the tumor-prone S47, an African-specific polymorphism of p53, is specifically impaired in its ability to transactivate a subset of target genes involved in ferroptotic responses, suggesting the potential relevance of ferroptosis to tumor suppression by p53 [103]. Together, these findings implicate that p53-mediated ferroptosis denote another critical layer of defense against tumorigenesis independently from cell cycle arrest, apoptosis, and senescence.

In fact, both DNA damage and intracellular ROS acquired from exogenous stress or endogenous cellular metabolism could promote tumor initiation and malignant transformation, it can also be detrimental in excess. The divergent effects of p53 in response to DNA damage and oxidative stresses suggest that p53 plays an intricate role in balancing cell survival and death to maintain cellular integrity and prevent tumor formation. In response to low levels of DNA damage and ROS, p53 promotes cell survival by inducing cell cycle arrest and alleviating ROS accumulation to repair both genotoxic and oxidative damage. While upon inappropriate levels of DNA damage and oxidative stress, p53 is activated to eliminate unsalvageable cancer cells through both apoptosis and ferroptosis. Notably, since ferroptosis is a fairly new field that was discovered recently, there is much yet to be uncovered regarding its molecular mechanism and unknown regulators. It is possible that additional components of the ferroptosis pathway involve

p53. Further studies are required to elucidate the regulation of ferroptosis by p53, and its role in p53-mediated tumor suppression.

1.6 Summary

Although p53 has been extensively studied over the past three decades, a better understanding of the non-canonical functions of p53 and their roles in mediating tumor suppression are further required. The experiments in this study were designed to expand the scope of p53 unconventional functions (including metabolic regulation and ferroptosis) through novel targets identification, and to explore their contribution to p53 as a tumor suppressor. In this study, we identified PHGDH and SAT1 as two novel metabolic targets of p53 that are involved in p53-mediated apoptotic and ferroptotic responses, respectively. We also uncovered two novel acetylation sites that are pivotal for the regulation of metabolic target genes (including *SAT1*, *SLC7A11*, and *TIGAR*), and revealed that p53-mediated ferroptotic response via SAT1 and SLC7A11 may denote another layer of defense against tumorigenesis together with other p53 functions.

CHAPTER 2

Identification of Phosphoglycerate Dehydrogenase (PHGDH)

as a novel p53 target in serine metabolism

2.1 Background and Rationale

Cancer cells are known to have altered metabolism. As described by Otto Warburg, the majority of tumor cells use aerobic glycolysis instead of mitochondrial respiration to produce energy and support cell growth and proliferation [104]. In addition, the metabolic reprogramming towards macromolecule biosynthesis, which is thought to sustain rapid cell growth, is also being recognized as a hallmark of cancer [68]. There is accumulating evidence indicating that p53 plays a critical role in modulating metabolism in human cancers [105]; however, the precise mechanisms by which the metabolic activities contribute to overall p53 responses are not well understood [106-109].

Recently, the serine synthesis pathway (SSP) has been shown to be essential in certain human cancers and contributes to oncogenesis through diverting glycolytic flux and further promote biosynthesis [110, 111]. L-serine is an important precursor for macromolecule biosynthesis. It is an essential amino acid for protein synthesis, as well as synthesis of sphingolipids and phospholipids, which are important components of cellular membranes [112, 113]. The serine synthesis pathway converts the glycolytic intermediate 3-phosphoglycerate into serine via a 3-step reaction involving three metabolic enzymes: phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1) and phosphate ester hydrolysis (PSPH) [114]. L-serine can then be converted into glycine by serine hydroxymethyltransferase (SHMT),

which results in the formation of one-carbon (1C) units that contribute to *de novo* synthesis of purines and pyridines [115]. Specifically, PHGDH catalyzes the first and rate-limiting step of SSP from 3-phosphoglycerate to phospho-hydroxypyruvate, and is potentially important during tumor development. The human *PHGDH* gene is located in a highly amplified region of chromosome 1p [110]. It is frequently amplified and over-expressed in 70% of the ER-negative breast cancers, 39% of melanoma, and cervical cancers [110, 111, 116, 117]. Depletion of PHGDH in *PHGDH* amplified cells leads to an impairment of cell proliferation and serine synthesis in both breast cancer and melanoma cell lines [110, 111, 118], suggesting that the repression of the serine synthesis pathway may have therapeutic roles toward suppressing certain types of cancers.

Cancer cells can acquire L-serine from both *de novo* synthesis and extracellular environment. Upon serine starvation, PHGDH and PSAT1 are significantly up-regulated, and thereby, increases conversion of 3-phosphoglycerate to serine [119]. Furthermore, serine starvation also leads to p53-dependent metabolic remodeling in cancer cells [119]. In this study, we further investigated the contribution of p53 to the regulation of serine metabolism. p53 is widely accepted as a tumor suppressor and responds to varieties of cellular stresses [120]. It mainly functions as a transcription factor to monitor signaling pathways through promoter-specific regulation of target genes involved in cell growth arrest, apoptosis and

senescence [121]. Recent studies have suggested that p53 can also regulate metabolic reprogramming through numerous target genes, which includes *TIGAR*, *GLUT1/3/4*, *GLS2*, *PARK3*, and so forth [70, 71, 73, 74, 105, 107, 122]. Therefore, further understanding of the regulation of serine metabolism pathway by p53 might be crucial for revealing its mechanisms in tumor suppression.

Although p53 is frequently mutated in human cancers, 80% of human melanomas retain wild-type p53 [109, 123]. Furthermore, PHGDH is frequently amplified and highly expressed in melanoma [111]. As such, we focused our study on the interaction between p53 and PHGDH in melanoma cell lines. Here, we find that *PHGDH* is a novel p53 repressing gene in the serine biosynthesis pathway. PHGDH expression is significantly down-regulated upon treatment of Nutlin-3, a p53 agonist that has been developed to suppress tumor growth through interrupting the interaction of p53 and its negative regulator Mdm2 [124]. Although Nutlin-3 only induces p53-mediated growth arrest, not apoptosis [125-127], in melanoma cell lines expressing wild-type p53, we observed that serine starvation further sensitizes cells to Nutlin-3-induced apoptosis in melanoma cells through repression of PHGDH by p53.

2.2 Materials and Methods

Plasmids

pGIPZ shRNA against TP53 and pTRIPZ shRNA against PHGDH were purchased from Thermo Scientific. A control hairpin in the pGIPZ vector that targeted *GFP* (shGFP) was used. pBabe-puro-FLAG-PHGDH was generated by PCR-based subcloning.

Cell culture and stable lines

All cells were cultured in 37°C incubator with 5% CO_2. All medium used were supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin (all Gibco). For serine starvation experiments, cells were washed with PBS twice and fed with serine and glycine free medium (consist of MEM (11095, Gibco) supplemented with additional 1× MEM vitamins (11120, Gibco), 10% dialyzed-FBS (26400, Gibco) and additional D-glucose to 25mM (25-037-Cl, Cellgro)). For control medium, serine and glycine (Sigma) were added back to a final concentration of 0.4mM. According to Maddocks *et al*., serine is the major factor in serine and glycine starvation, therefore all starvation experiments were described as serine starvation [119]. To generate A375 cell line with stable knock-down of p53, HEK293T cells were transfected with pGIPZ shRNA vectors against TP53 and lenti-viral packaging vectors, and lenti-viruses produced from 293T were used to infect A375 cells. Selection under 1 μg/mL puromycin was carried out two days after infection. A375-PHGDH

stable cell line and A375 inducible knock-down of PHGDH cell line (A375-shPHGDH) were generated by similar procedure with pBabe-puro-FLAG-PHGDH and pTRIPZ-shPHGDH vector. To induce knock-down of PHGDH, 5 μg/mL of doxycycline (Sigma) were added to culture media.

siRNA-mediated ablation of ATF4

Knock-down of ATF4 was performed by transfection of A375-shPHGDH cells with siRNA duplex oligoset (On-Target-Plus Smartpool L-00512500, Dharmacon) using Lipofectamine RNAiMAX (13778030, Invitrogen) according to manufacturer's protocol. Control siRNA (On-Target-Plus siControl nontargeting pool D00181010, Dharmacon) was also used for transfection.

Western blotting and antibodies

Cell lysates were prepared in Flag lysis buffer with fresh protease inhibitor cocktail. Protein extracts were analyzed by western blotting according to standard protocols using primary antibodies specific for PHGDH (HPA021241, Sigma), p53 (human: DO-1, Santa Cruz), Mdm2 (Ab5, Millipore), Tigar (E-2, Santa Cruz), p21 (SX118, Santa Cruz), Puma (H-136, Santa Cruz), Cleaved-caspase3 (Asp175, Cell Signaling Technologies), ATF4 (sc-200, Santa Cruz) and β-Actin (A3853, Sigma-Aldrich). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (GE Healthcare) were used and signals were detected on autoradiographic films with ECL western blotting detection system (GE Healthcare) or SuperSignal West Dura reagents

(Thermo scientific).

RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer's protocol. cDNA was synthesized from total RNA using M-MulV Reverse Transcriptase kit (NEB). PCR analysis was performed using Applied Biosystems 7500 Fast System. For the qRT-PCR analysis of human transcripts the following primers were used: *PHGDH* forward 5'-ATCTCTCACGGGGGTTGTG-3',

PHGDH reverse 5'-AGGCTCGCATCAGTGTCC-3',

ATF4 forward 5'-GGTCAGTCCCTCCAACAACA-3',

ATF4 reverse 5'-CTATACCCAACAGGGCATCC-3',

PUMA forward 5'-GGTCCTCAGCCCTCGCTCTC-3',

PUMA reverse 5'-CTTGTCTCCGCCGCTCGTAC-3',

NOXA forward 5'-CGTGTGTAGTTGGCATCTCCG-3',

NOXA reverse 5'-GACGCGAGCTGAACACGAAC-3',

BAX forward 5'-TTCTGACGGCAACTTCAACTGG-3',

BAX reverse 5'-CCCGGAGGAAGTCCAATGTC-3',

GAPDH forward 5'-ATCAATGGAAATCCCATCACCA-3',

GAPDH reverse 5'-GACTCCACGACGTACTCAGCG-3'.

Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed in transfected H1299 cells or A375 cells as previously described [128]. Primers used for p53 ChIP assay are:

PHGDH forward 5'-TGAGAATATGCGGTGTTTGG-3',

PHGDH reverse 5'-GGGTAAATGTGCAAGGCACT-3',

TIGAR forward 5'-CGGCAGGTCTTAGATAGCTT-3',

TIGAR reverse 5'- GGCAGCCGGCATCAAAAACA-3'.

Electrophoretic mobility shift assay (EMSA)

Purified Flag-p53 protein was obtained from transfected H1299 cells. The 234-bp DNA probe containing p53 responsive elements was PCR-amplified from *PHGDH* promoter using PHGDH ChIP primers, labeled by T4 kinase (NEB, M0201S) and purified using the Bio-Spin 30 columns (Bio-Rad). The protein-DNA binding reactions were performed as previously described [128]. In supershift assays, 100 ng pAb421 (Millipore) antibody were added to the reactions.

Cell death count, drugs and inhibitors

Cells were trypsinized, collected and stained with trypan blue, followed by counting with a hemocymeter using standard protocol. Cells stained blue under the microscope were considered as dead cells. Nutlin-3 (Sigma) was used in experiments at a concentration of 10 μM, except otherwise indicated. DNA-damaging agent Doxorubicin (Dox, Sigma) was used at 0.2 μg/ml.

Specific cell death inhibitors were used in the experiments with indicated concentrations as listed: Z-VAD-fmk (caspase 3 inhibitor, Sigma), 10 μg/mL; 3-MA (autophagy inhibitor, Sigma), 2mM; Necrostatin-1 (necroptosis inhibitor, Sigma), 10 μg/mL; and Ferrostatin-1 (Ferroptosis inhibitor, Xcess Biosciences), 2 μM.

Metabolite measurements

To measure steady-state metabolite levels, pre-chilled (on dry ice) 80% methanol was added onto cells in 10-cm culture dishes followed by incubation on dry ice for 15 min. Cells were then collected by scraping, and cellular metabolite was extracted by collecting the supernatant following centrifugation at 4,000 r.p.m for 10 min (4°C). The supernatant was then dried down using lyophilizer (Labconco) and stored at -80°C before subjected for LC-MS/MS based metabolite analysis at the Beth Israel Deaconess Medical Center Mass Spectrometry Core Facility as previously described [129]. Briefly, samples were suspended in HPLC grade water and analyzed using a 5500 QTRAP hybrid triple quadrupole mass spectrometer (AB/SCIEX) coupled to a Prominence UFLC HPLC system (Shimadzu) via selected reaction monitoring of a total of 254 endogenous water soluble metabolites using positive and negative polarity switching. Peak areas from the total ion current for each metabolite were integrated using MultiQuant v2.0 software (AB/SCIEX). Three biological triplicates were analyzed for each treatment. In addition, two parallel dishes of cells were collected and measured for protein concentration using Bradford

assay (Bio-Rad). Subsequent metabolite measurements were normalized to total protein amount.

2.3 Results and Discussion

2.3.1 Nutlin-3 induces p53-mediated repression of PHGDH

To evaluate the regulation of PHGDH by endogenous p53, we analyzed human melanoma cell lines containing wild-type p53 or mutant p53. Wild-type p53 cell lines (A375, Colo-829 and WM26-64) and mutant p53 cell lines (Colo-800 and Sk-mel28) were treated with Nutlin-3, a non-genotoxic drug that activates p53 by interrupting Mdm2-p53 interaction. Total cell lysates were then harvested and subjected to western blot analysis, and mRNA was extracted for quantitative real-time PCR (qRT-PCR). As expected, in p53-wildtype cell lines, the protein levels of p53, as well as its transcriptional targets Mdm2 and p21, were increased in response to Nutlin-3 treatment (Figure 2.1A). Notably, a significant decrease in both PHGDH protein and mRNA levels were also observed (Figure 2.1). However, we did not observe a change in PHGDH expression levels in p53-mutant melanoma cell lines after the same treatment (Figure 2.1). To further determine p53 dependent regulation on PHGDH expression, we compared A375 cells with p53-depletion to those without. Cells that underwent mock depletion and treated with Nutlin-3 showed a time-dependent reduction of both PHGDH protein and mRNA levels (Figure 2.2). In contrast, the expression of PHGDH in p53-depleted cells remained unchanged after Nutlin-3 treatment (Figure 2.2). Together, these results indicate that PHGDH is transcriptionally

down-regulated upon Nutlin-3 treatment in a p53-dependent manner.

Figure 2.1

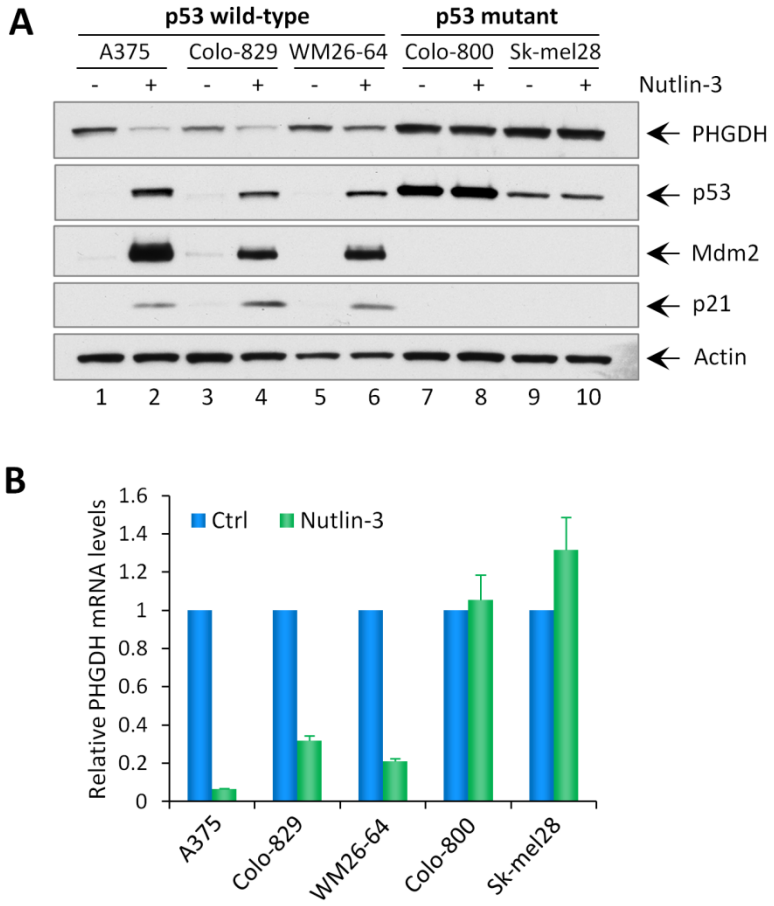

Figure 2.1. Nutlin-3 induces p53-mediated repression of PHGDH. (A) Western blot analysis of the protein levels of PHGDH, p53, Mdm2, p21 and Actin in indicated melanoma cell lines (A375, Colo-829 and WM26-64: p53 wild-type; Colo-800 and Sk-mel28: p53 mutant) without (-) or with (+) Nutlin-3 treatment (10μM) for 2 days. **(B)** The mRNA expression levels of PHGDH in the indicated melanoma cell lines treated with Nutlin-3 (10μM) were measured using qRT-PCR (bar graph). All mRNA expression levels were normalized with GAPDH. Error bars in panel A and B represent s.d. from three experiments.

Figure 2.2

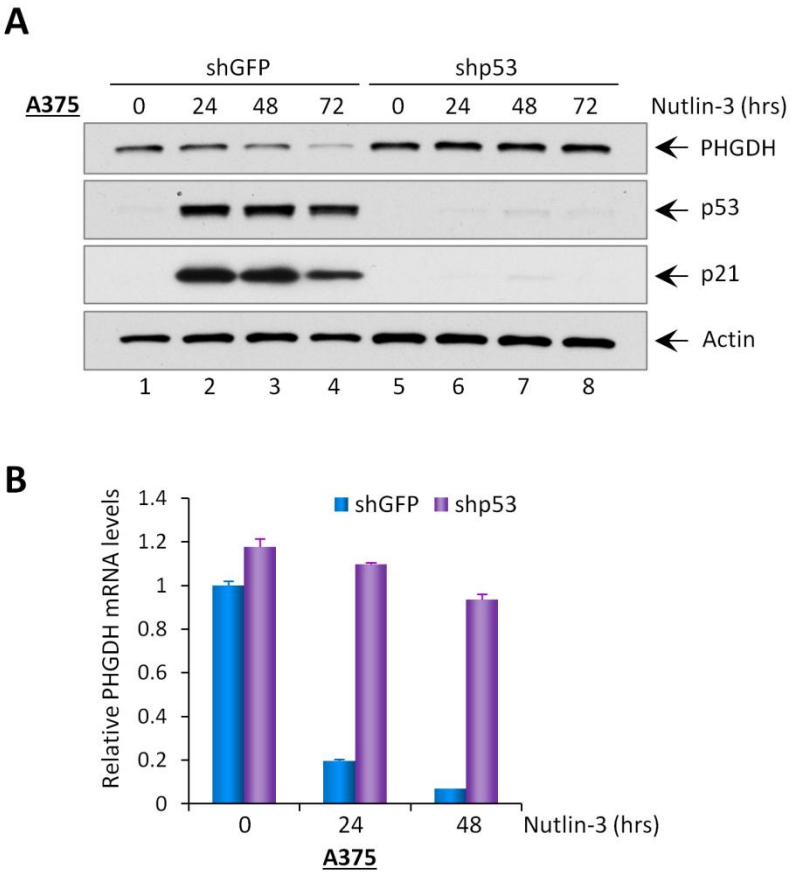

Figure 2.2. Nutlin-3-induced repression of PHGDH is p53-dependent. (A) A375-shGFP and A375-shp53 stable knock-down cell lines were treated with Nutlin-3 (10μM) for the indicated times and total protein lysates were subjected to western analysis for the expression of PHGDH, p53, p21 and Actin. **(B)** qRT-PCR analysis of PHGDH mRNA levels in A375-shGFP and A375-shp53 cell lines treated with Nutlin-3 (10μM). All mRNA expression levels were normalized with GAPDH. Error bars in panel A and B represent s.d. from three experiments.

2.3.2 DNA damage down-regulates PHGDH protein and mRNA levels in a p53-dependent manner

To further confirm that *PHGDH* is a p53-repressing target, we examined PHGDH expression in response to DNA damage. As expected, DNA-damaging agent Doxorubicin (Dox) treatment induced activation of p53 (demonstrated by up-regulation of its downstream targets, p21) in p53-wildtype cell lines, but not in p53-mutant cell lines (Figure 2.3A). Notably, in all three p53-wildtype melanoma cell lines, the levels of PHGDH protein and mRNA were decreased upon Doxorubicin treatment (Figure 2.3). In contrast, PHGDH expression remained unchanged in p53-mutant melanoma cell lines, despite undergoing the same treatment (Figure 2.3). Similarly, A375 cells that underwent mock depletion and treated with Doxorubicin showed a time-dependent reduction of both PHGDH protein and mRNA levels. However, the expression of PHGDH was not affected upon the same treatment in p53-depleted cells (Figure 2.4). These data collectively suggest that the expression of PHGDH is transcriptionally repressed by p53, upon p53 stabilization after treatment with DNA-damaging agents.

Figure 2.3

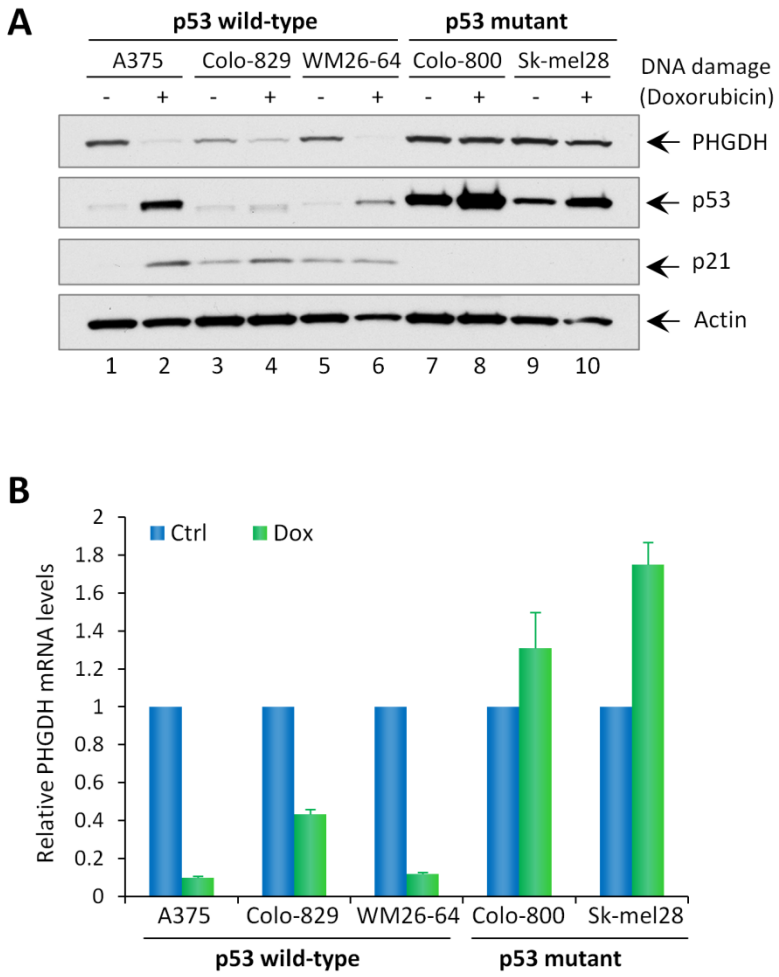

Figure 2.3. DNA damage down-regulates PHGDH protein and mRNA levels in a p53-dependent manner. (A) Expression of endogenous PHGDH, p53, p21 and Actin in the indicated melanoma cell lines without (-) or with (+) DNA-damaging agent Doxorubicin treatment (0.2μg/mL). **(B)** qRT-PCR analysis of PHGDH mRNA levels in the indicated melanoma cell lines treated with Doxorubicin (Dox, 0.2μg/mL). All mRNA expression levels were normalized with GAPDH. Error bars represent s.d. from three experiments.

Figure 2.4

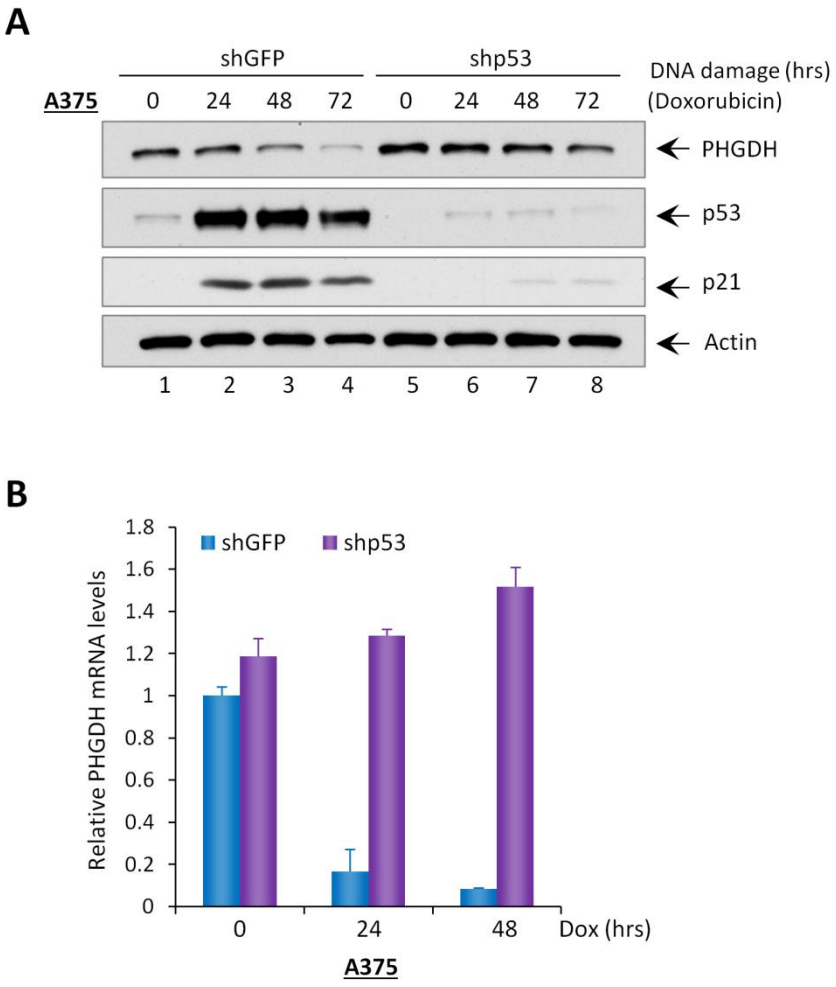

Figure 2.4. DNA damage-induced down-regulation of PHGDH is p53-dependent. (A) A375-shGFP and A375-shp53 stable knock-down cell lines were treated with Doxorubicin (0.2µg/mL) for the indicated times and total protein lysates were analyzed by western blotting for the expression of PHGDH, p53, p21 and Actin. **(B)** qRT-PCR analysis of PHGDH mRNA levels in A375-shGFP and A375-shp53 cell lines treated with Doxorubicin (0.2µg/mL) for indicated times. All mRNA expression levels were normalized with GAPDH. Error bars represent s.d. from three experiments.

2.3.3 *PHGDH* promoter contains one p53 binding site

As a transcription factor, p53 binds to its responsive elements (REs) in its target genes and regulates gene transcription in response to various stresses. As shown in Figure 2.5A, we found a consensus binding sequence in promoter region of the *PHGDH* gene that matches with p53 binding site sequence [130]. To verify that p53 binds to its responsive element in the promoter region of the *PHGDH* gene, chromatin immunoprecipitation (ChIP) assays were performed. H1299 p53-null cells were transfected with p53 expression vector, cross-linked with formaldehyde, and p53-bound DNA were immunoprecipitated via anti-p53 antibody (α-p53). Resulting DNA fragments were amplified using primers flanking the predicted p53 binding site in the *PHGDH* promoter. In this assay, we observed enrichment of PHGDH and TIGAR promoter sequences in the presence of p53 (Figure 2.5B). Furthermore, binding of p53 at the *PHGDH* promoter was significantly enriched in A375 cells treated with either Nutlin-3 or Doxorubicin (Figure 2.6A). These results demonstrate that p53 interacts with the p53 responsive element in the *PHGDH* promoter in *vivo*.

We next performed electrophoretic mobility shift assay (EMSA) to determine whether p53 directly binds to *PHGDH* promoter in *vitro*. Notably, a p53/DNA complex was identified upon incubation of radiolabeled oligonucleotide probes containing the responsive element with purified full-length human p53 protein (Figure 2.6B). Moreover, the p53/DNA complex was

enhanced and super-shifted in the presence of anti-p53 antibody (ab421) (Figure 2.6B). Furthermore, binding of p53 to the radiolabeled fragments was diminished and out-competed by the addition of unlabeled probes (Figure 2.6B). Together, these results indicate that the *PHGDH* gene is a transcriptional target of p53 and that the consensus p53 binding site on the *PHGDH* promoter is responsible for p53-dependent transcriptional repression.

Figure 2.5

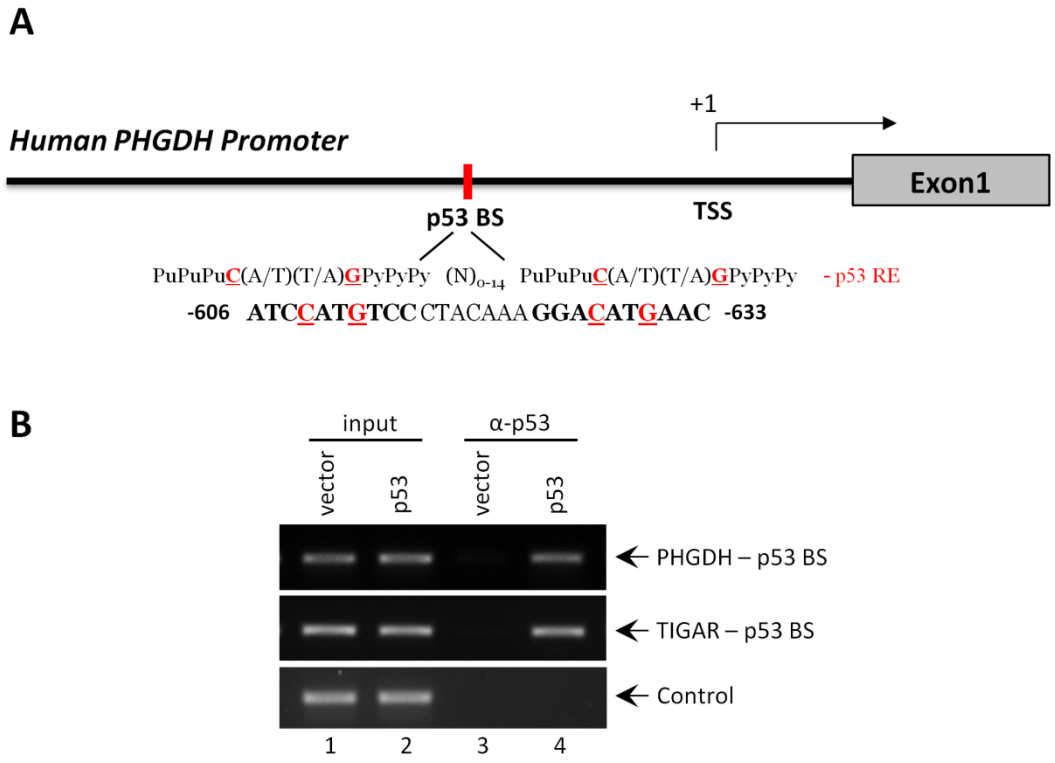

Figure 2.5. *PHGDH* promoter contains one p53 binding site. (A) Schematic representation of the promoter region in human *PHGDH* gene. The p53 binding site upstream of the first exon is indicated and compared with consensus p53 responsive element (p53 RE). Pu, purine; Py, pyramidine; N, any nucleotide. **(B)** ChIP-semi-quantitative analysis of p53 enrichment at the promoter regions of *PHGDH* and *TIGAR* in H1299 cells transfected with empty or p53 vector for 24 hours. Cross-linked protein/DNA complex was immunoprecipitated using full-length anti-p53 antibody (α-p53). *GAPDH* was used as a negative control.

Figure 2.6

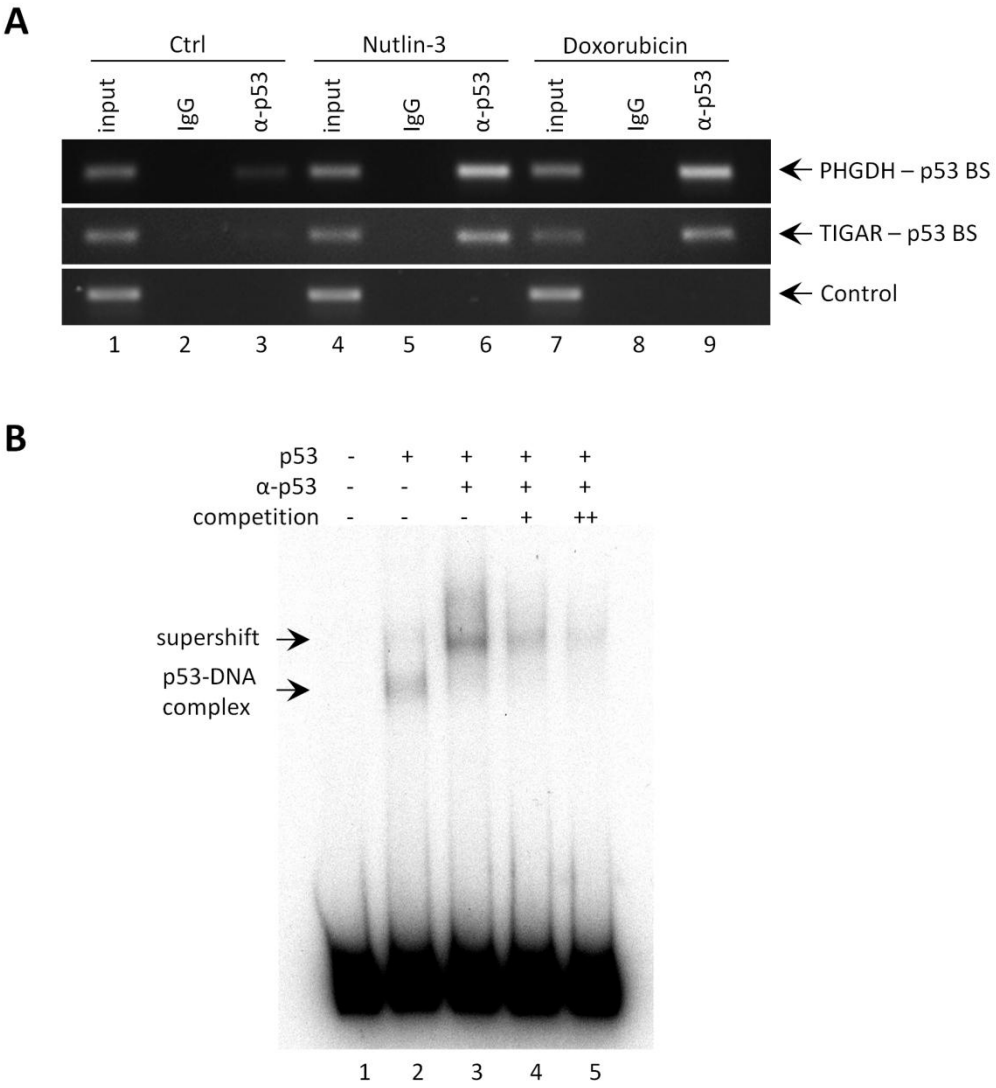

Figure 2.6. p53 binds to *PHGDH* promoter both *in vitro* and *in vivo*. (A) A375 cells were treated with vehicle, Nutlin-3 (10μM) or Doxorubicin (0.2μg/mL) for 24 hours, and ChIP assays for determining the enrichment of p53 on *PHGDH* and *TIGAR* promoters were carried out using anti-p53 antibody (α-p53) or control rabbit IgG. *GAPDH* was used as a negative control. **(B)** EMSA showing the binding of p53 on ^{32}P-labeled oligonucleotides containing the p53-binding site in the human *PHGDH* promoter region. The DNA binding activity of purified p53 protein was enhanced with C-terminal p53 antibody pAb421 (α-p53). The binding specificity was verified using an excess of non-radiolabeled wild-type probes (competition).

2.3.4 Serine starvation promotes Nutlin-3-induced cell death through repression of PHGDH

As a non-essential amino acid, serine can be acquired through *de novo* serine synthesis, as well as from exogenous nutrient source. Since serine is an important building block for biosynthesis and contributes to cancer cell growth, our findings that PHGDH is a p53 target suggest that PHGDH may mediate p53 function in controlling cell fate upon serine starvation. Previous studies have shown that treatment of Nutlin-3 on melanoma cell lines expressing wild-type p53 only induces p53-mediated cell cycle arrest but not apoptosis [125-127]. As expected, A375 cells (p53-wildtype) treated with Nutlin-3 only showed significant growth arrest with modest increase in cell death (Figure 2.7). Surprisingly, although serine starvation alone did not induce obvious growth arrest nor cell death, combination of serine starvation and Nutlin-3 treatment induced significant cell death in A375 cells (Figure 2.7). Notably, Puma, a major p53 up-regulated apoptosis modulator, was further activated upon combination treatments as compared to Nutlin-3 treatment alone, while p53 levels were the same in these two conditions, indicating that serine deprivation may lead to p53-independent Puma activation (Figure 2.8A). To ascertain whether the cell death is dependent on p53, p53-depleted A375 cells and p53-mutant melanoma cell lines (Colo-800 and Sk-mel28) were treated with Nutlin-3 and/or serine starvation. Notably, Nutlin-3 did not induce growth arrest nor cell death upon serine starvation in these cell lines (Figure 2.8B and 2.8C), indicating that Nutlin-induced cell death upon serine starvation is

Figure 2.7

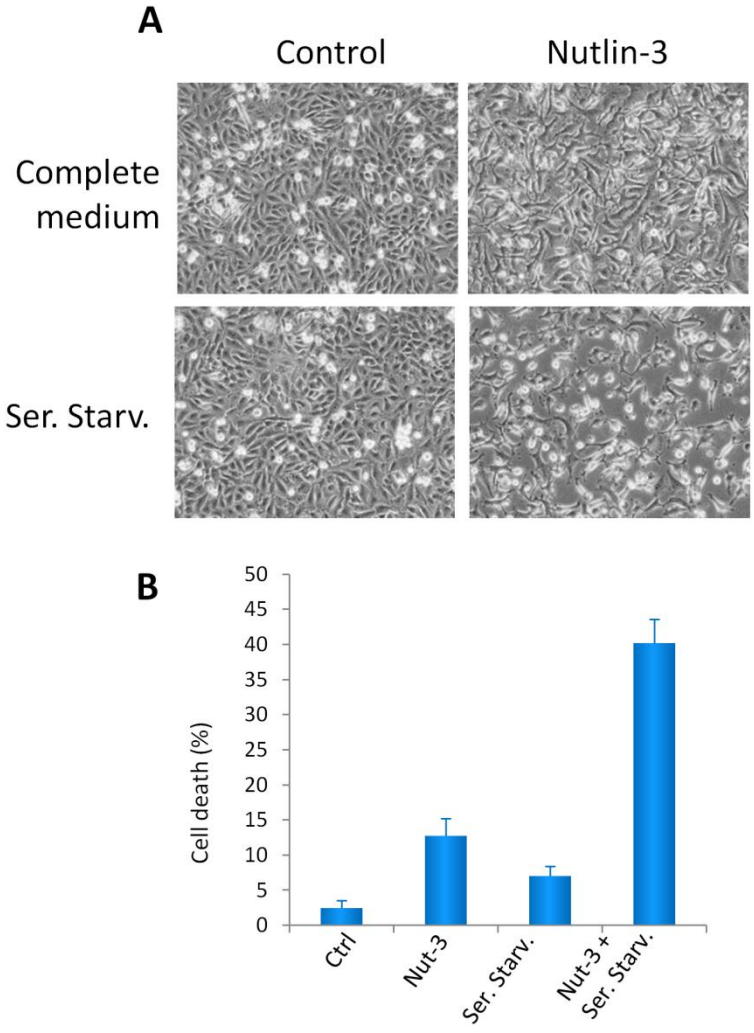

Figure 2.7. Serine starvation sensitizes Nutlin-3 to induce cell. (A) Representative phase-contrast images of A375 cells treated with Nutlin-3 (10μM) with the presence (Complete medium) or absence (Ser. Starv.) of serine in culture medium. **(B)** The percentages of cell death for all treatments shown in A were quantified by trypan blue exclusion assay.

Figure 2.8

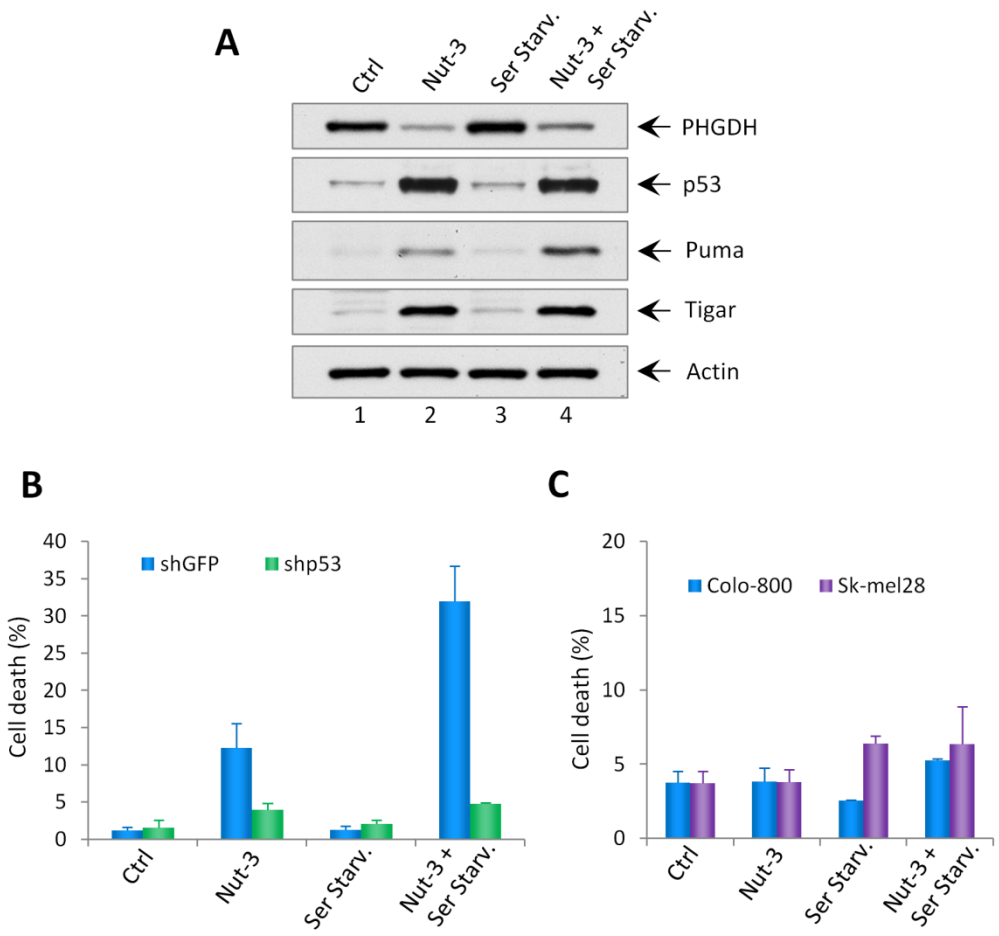

Figure 2.8. Nutlin-induced cell death upon serine starvation is p53-dependent. (A) Western blot analysis of expression levels of PHGDH, p53, Puma, Tigar and Actin in A375 cells treated with Nutlin-3 (10μM) with the presence or absence of serine. **(B)** The percentages of cell death in A375-shGFP and A375-shp53 stable knock-down cells treated with Nutlin-3 (10μM) in the presence or absence of serine. **(C)** The percentages of cell death in p53 mutant cell lines (Colo-800 and Sk-mel28) treated with Nutlin-3 (10μM) in the presence or absence of serine.

p53-dependent.

To further explore the effect on cell fate by p53-mediated repression of PHGDH, we generated a PHGDH over-expression stable cell line in A375 (A375-PHGDH). As shown in Figure 2.9A, while the expression of PHGDH was decreased upon Nutlin-3 treatment in A375 cells, it remained unchanged and over-expressed in A375-PHGDH cells after the same treatment, indicating that the high expression level of exogenous PHGDH negates the repressive effect of p53 on endogenous PHGDH expression. As a result, serine starvation failed to sensitize A375-PHGDH cells to Nutlin-3-induced cell death (Figure 2.9B), indicating that the cell death induced by the combination of Nutlin-3 and serine starvation is mediated through repression of PHGDH by p53. Together, our results indicate that the function of p53 in repressing PHGDH mediates its role in regulating cell fate upon serine starvation.

Figure 2.9

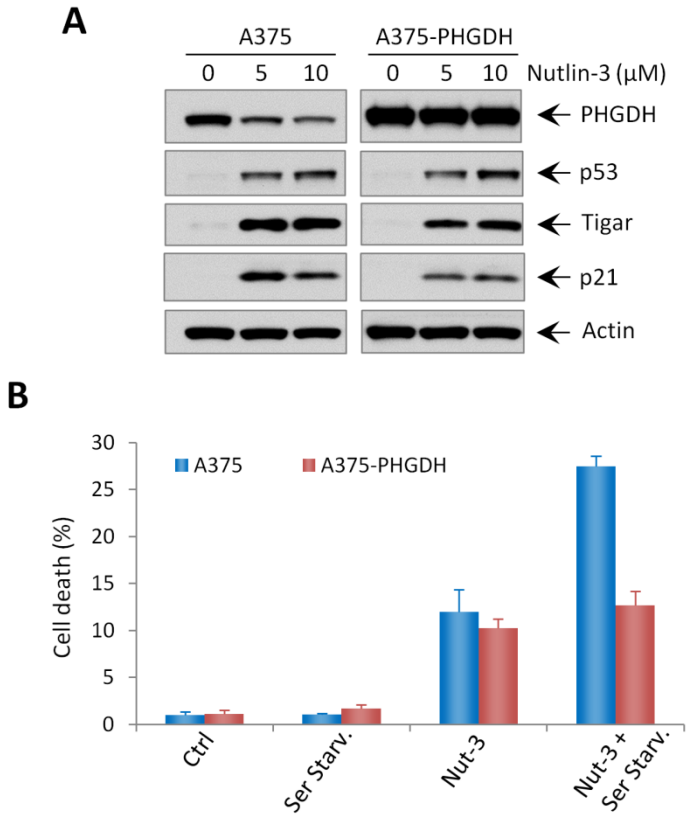

Figure 2.9. Serine starvation sensitizes Nutlin-3 to induce cell death through repression of PHGDH. (A) A375 and A375-PHGDH cell lines were treated with the indicated concentrations of Nutlin-3 for 48 hours, and the expression levels of PHGDH, p53, Tigar, p21 and Actin were measured by western blotting. **(B)** The percentages of cell death in A375 and A375-PHGDH cell lines treated with Nutlin-3 (10μM) under normal or serine starvation condition. All error bars represent s.d. from three different experiments.

2.3.5 Mechanistic insights into the apoptotic response regulated by PHGDH upon serine starvation

To understand the mechanisms of how serine starvation sensitizes cells to cell death upon Nutlin-3 treatment in p53-wildtype melanoma cell lines, we investigated whether PHGDH depletion is sufficient to induce cell death upon serine removal. As such, a doxycycline-inducible shRNA cell line in A375 targeting PHGDH (Tet-on A375-shPHGDH) was generated. As shown in Figure 2.10A, PHGDH expression was efficiently depleted upon addition of doxycycline. Cells that underwent mock depletion or PHGDH depletion were then incubated in control medium or serine starvation medium for an additional 48 hours. Notably, PHGDH depletion in A375 cells induced significant cell death upon serine starvation, while PHGDH depletion or serine starvation alone did not affect cell survival (Figure 2.10B and 2.11A). Interestingly, the cell death induced by PHGDH depletion and serine starvation was completely rescued by caspase-3 inhibitor Z-VAD-fmk, but not inhibitors of other forms of cell death, including autophagy (3-MA), necroptosis (Necrostatin-1) and ferroptosis (Ferrostatin-1) (Figure 2.10B and 2.11A). Western blot analysis also showed elevated levels of cleaved-caspase-3 and Puma in cells that were undergoing cell death (Figure 2.11B). These results indicate that the synergistic effect of PHGDH depletion and serine starvation leads to apoptosis in A375 cells.

Figure 2.10

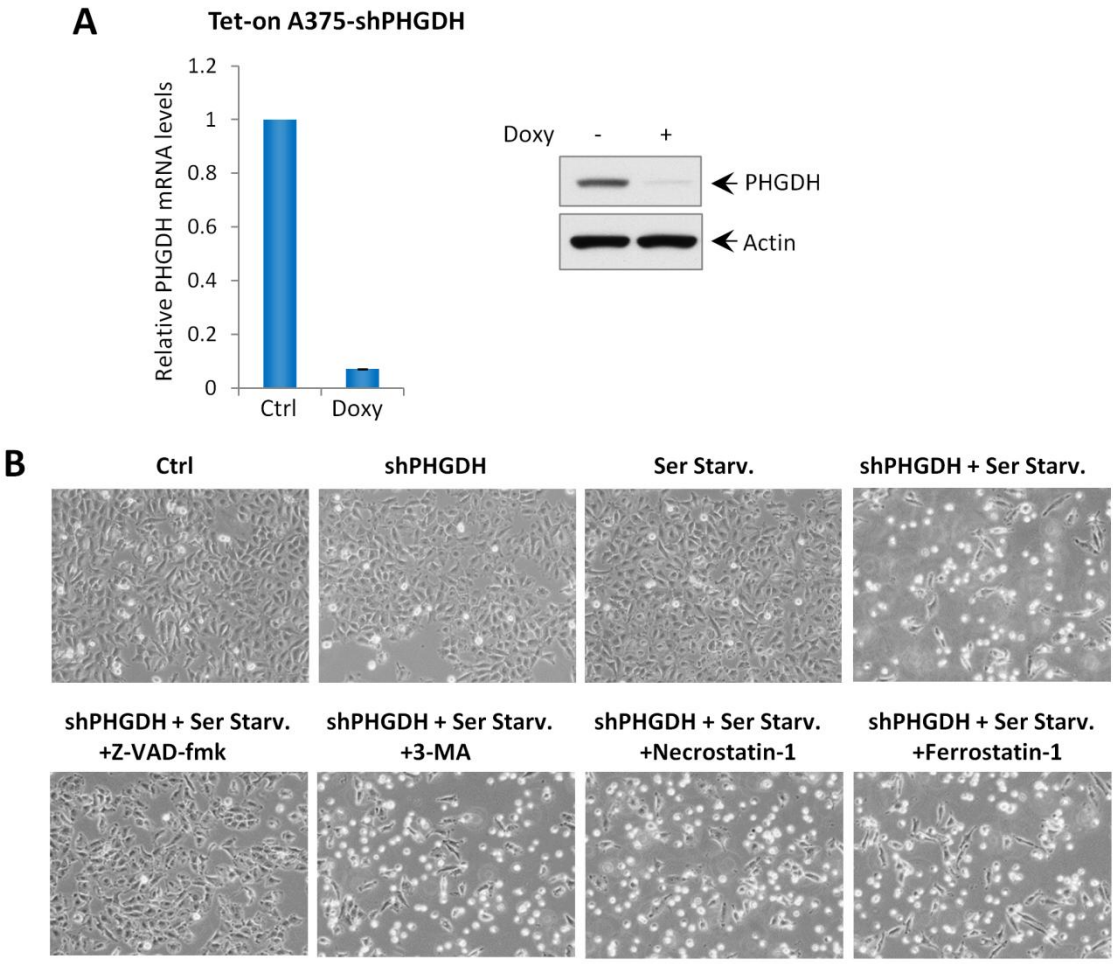

Figure 2.10. PHGDH knock-down induces apoptosis upon serine starvation. (A) qRT-PCR analysis of PHGDH mRNA levels in Tet-on A375-shPHGDH stable cell line after adding doxycycline (Doxy, 5μg/mL) for 3 days. Western blot analysis also showing the expression levels of PHGDH and Actin in Tet-on A375-shPHGDH cells after adding doxycycline. **(B)** Representative phase-contrast images of A375 cells expressing doxycycline-inducible shRNA against PHGDH, with or without serine starvation (Ser Starv.) for 48 hours. Images also showing A375-shPHGDH cells under serine starvation condition with the addition of cell death inhibitors (Z-VAD-fmk, caspase-3 inhibitor, 10μg/mL; 3-MA, autophagy inhibitor, 2mM; Necrostatin-1, necroptosis inhibitor, 10μg/mL and Ferrostatin-1, ferroptosis inhibitor, 2μM).

Figure 2.11

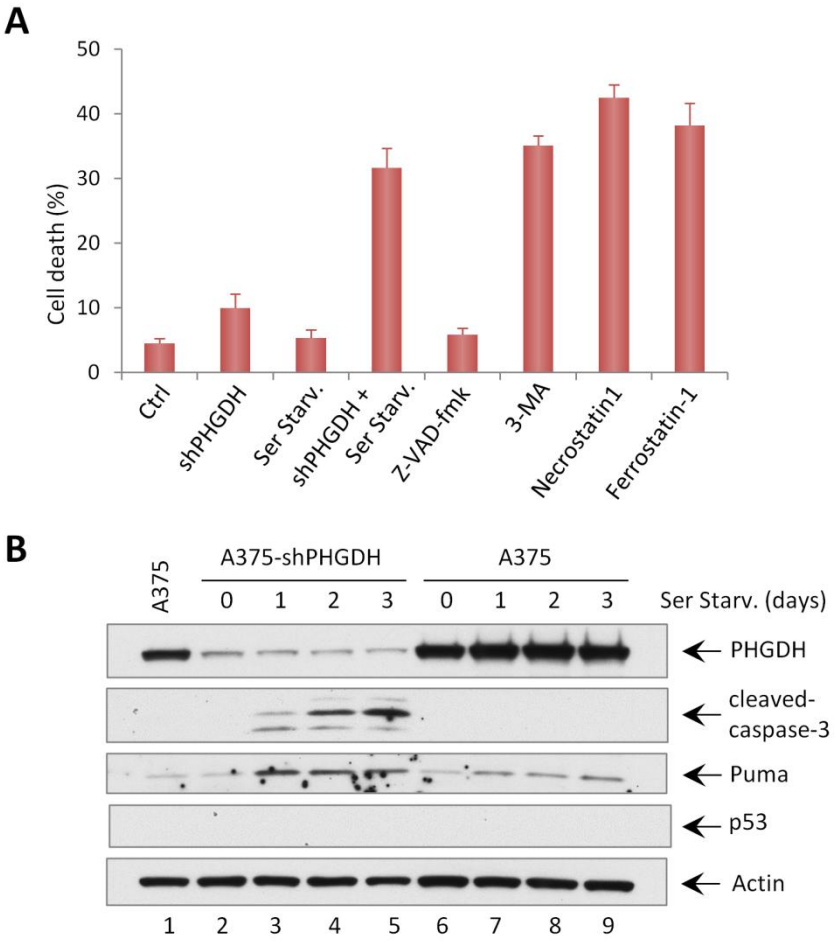

Figure 2.11. PHGDH knock-down induces apoptosis upon serine starvation (continued). (A) The percentages of cell death for all experiments shown in Figure 2.10B were measured by trypan blue exclusion assay. **(B)** A375 shPHGDH cells and A375 cells were incubated in serine starvation medium for the indicated times and total protein lysates were subjected to western analysis for the expression of PHGDH, Cleaved-caspase3, Puma, p53 and Actin.

Although the link between apoptotic pathways and amino acid starvation is not fully understood, recent studies suggested that activation transcription factor 4 (ATF4) plays an important role in regulating amino acid metabolism and tumor cell death under stress conditions [131]. Qing *et al*. found that glutamine starvation induces apoptosis through ATF4-dependent, but p53-indepdent, Puma and Noxa induction in *MYC*-transformed cells [131]. In our study, metabolite analysis showed that steady-state serine levels were markedly decreased in PHGDH-depleted A375 cells upon serine starvation (Figure 2.12A). Meanwhile, a significant up-regulation of ATF4, Puma and Noxa levels, and a slight induction of apoptosis regulator Bax were observed in A375 cells undergoing apoptosis (Figure 2.12B). However, no p53 activation was present in this condition (Figure 2.11B). To further prove that PHGDH knock-down induced apoptosis upon serine starvation is indeed dependent on ATF4, PHGDH-depleted A375 cells were transfected with control siRNA or ATF4 siRNA, and then incubated in control medium or serine-free medium. While ATF4 expression was significantly up-regulated upon serine starvation in PHGDH knock-down A375 cells, its expression was completely ablated upon ATF4 knock-down (Figure 2.13A). Notably, activation of Puma was abolished and lower levels of cleaved-caspase-3 were observed upon ATF4 knock-down, while no p53 activation was present under any conditions (Figure 2.13A). Furthermore, ATF4 siRNA significantly reduced serine starvation-induced cell death in PHGDH-depleted A375 cells (Figure 2.13B). These results

collectively indicate that the apoptosis induced by PHGDH depletion upon serine starvation is dependent on ATF4.

Figure 2.12

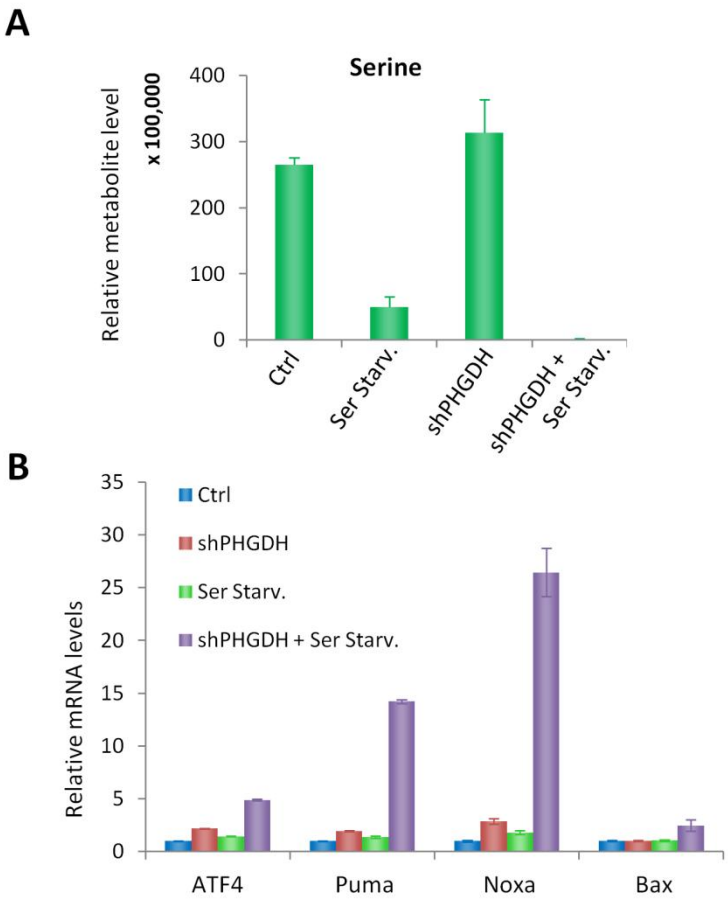

Figure 2.12. PHGDH knock-down induced depletion of cellular L-serine and activation of ATF4 upon serine starvation. (A) Metabolite analysis showing the intracellular steady levels of serine in A375-shPHGDH cells cultured in complete medium or serine starvation medium (Ser Starv.). **(B)** qRT-PCR analysis of mRNA levels of ATF4, Puma, Noxa and Bax in A375-shPHGDH cells cultured in control medium or serine starvation.

Figure 2.13

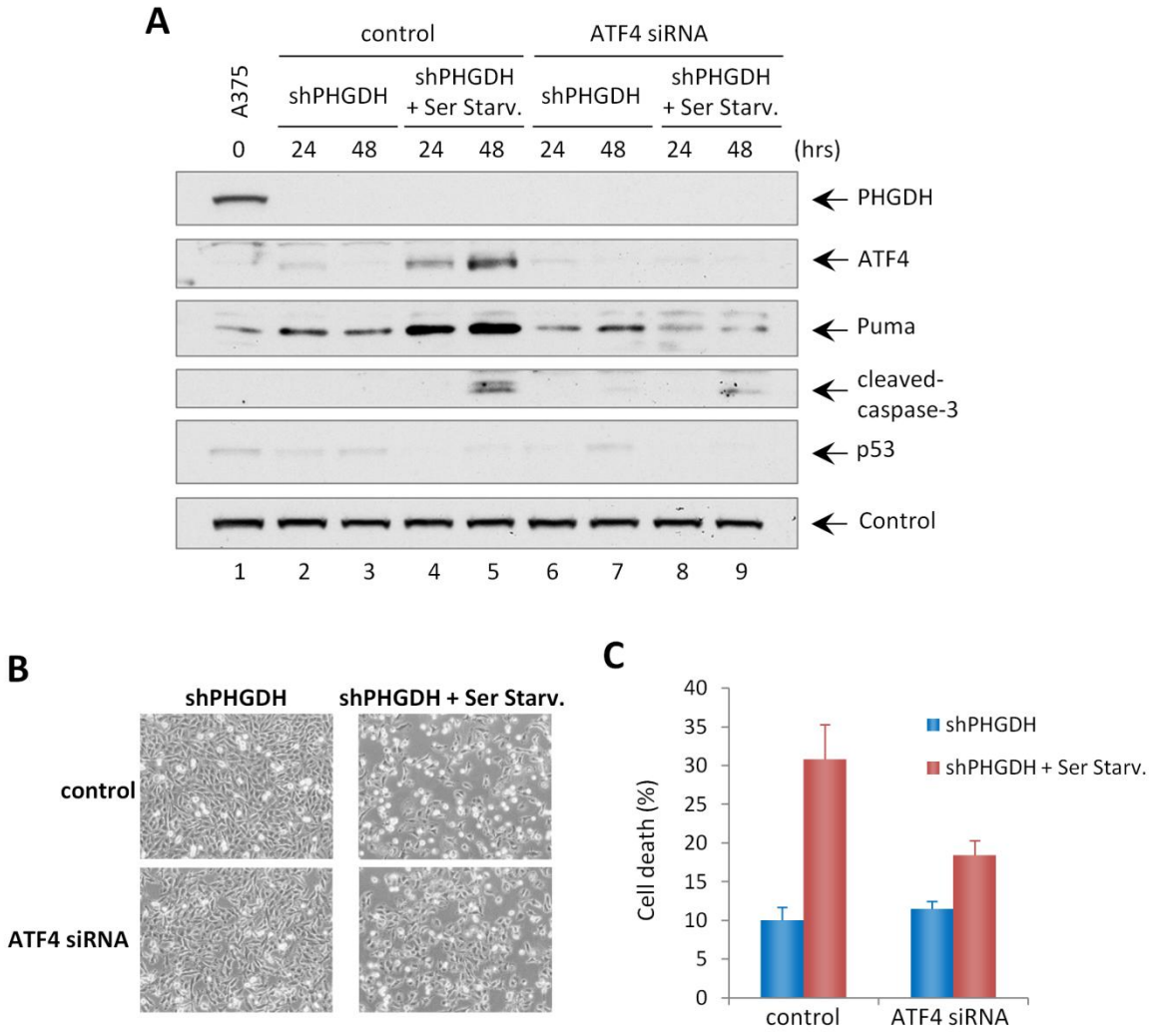

Figure 2.13. ATF4 mediates PHGDH knock-down induced apoptosis upon serine starvation. **(A)** A375 shPHGDH cells were first transfected with control siRNA or ATF4 siRNA for 24 hours, and then incubated in complete medium or serine starvation medium for up to 48 hours. Total cell lysates were harvested and subjected to western analysis for the expression of PHGDH, ATF4, Puma, Cleaved-caspase3 and p53. Vinculin was used as loading control. **(B)** Representative phase-contrast images of A375-shPHGDH cells transfected with control or ATF4 siRNA in the presence or absence of serine at 48 hours. **(C)** The percentages of cell death were measured by trypan blue exclusion assay. Error bars represent s.d. from three experiments.

CHAPTER 3

Identification of Spermidine/spermine N^1-acetyltransferase 1 (SAT1) as a novel p53 target in polyamine metabolism and ferroptosis

3.1 Background and Rationale

Recently, the identification of a novel p53 target SLC7A11 in cystine uptake has highlighted the importance of ferroptosis in p53-mediated tumor suppression [81]. Ferroptosis is an iron-dependent non-apoptotic cell death that can be triggered by inhibition of cystine uptake, decrease of glutathione (GSH) synthesis, and subsequent accumulation of lipid ROS [84]. Jiang *et al.* reported that in response to inappropriate levels of ROS, p53 promotes ferroptosis through down-regulation of SLC7A11, a component of the cystine/glutamate antiporter (system x_c^-), and therefore, provides another layer of defense against cellular injury and tumorigenesis [81]. Nonetheless, it is possible that additional p53 targets may also contribute to this novel p53 response. Therefore, further investigation is required to demonstrate the role of other metabolic targets of p53 in regulating ferroptotic cell death. In this section, we used RNA sequencing technique to search for novel metabolic targets of p53 in a p53 wild-type melanoma cell line A375 treated with Nutlin, a non-genotoxic drug that is commonly used to activate p53 through inhibiting its negative regulator MDM2 [132]. Our analysis identified a novel gene *SAT1* in the polyamine metabolism pathway that is highly induced by p53.

The polyamines are amino-acid-derived polycationic alkylamines that are essential for the growth and survival of eukaryotic cells [133]. They consist of putrescine, spermidine and spermine, and their levels are tightly controlled and regulated by enzymes involving polyamine

biosynthesis, catabolism and transport [134]. Notably, Scuoppo *et al.* reported that deletion of genes in polyamine-hypusine axis results in increased tumor formation in a mouse lymphoma model, implicating the role of polyamine metabolism in modulating tumorigenesis [135]. Furthermore, polyamine metabolism is found to be frequently dysregulated in cancers, thus making it an attractive target for therapeutic intervention [134, 136]. Spermidine/spermine N^1-acetyltransferase 1 (SAT1) is the rate-limiting enzyme controlling the first intracellular pathway of polyamine catabolism. As shown in Figure 3.1, SAT1 catalyses the acetylation of spermidine and spermine to form N^1-acetylspermidine and N^1-acetylspermine, which are then either exported from the cells, or converted back to putrescine or spermidine by N^1-acetylpolyamine oxidase (PAO) [137]. Therefore, overexpression of SAT1 leads to an overall depletion of spermidine and spermine, while increasing the levels of putrescine, N^1-acetylspermidine and N^1-acetylspermine [138]. In fact, N^1, N^{11}-di(ethyl)norspermine (DENSpm), a polyamine analogue that is known to induce SAT1, has been applied in several clinical trials in patients with advanced malignancies [139-142]. Nonetheless, it remains largely unknown whether SAT1 has a role in tumor suppression, and the molecular mechanism of polyamine metabolism in modulating tumorigenesis is not well understood either.

In this study, we identified *SAT1* as a novel p53 metabolic target gene that can be induced by both endogenous and exogenous p53. Expression of SAT1 in xenograft cells significantly

impaired tumor growth, indicating that it acts as a tumor suppressor *in vivo*. Surprisingly, we also discovered that SAT1 is involved in regulating p53-mediated ROS response and ferroptosis. These findings further broadened our understanding of the complex regulation of ferroptotic cell death, as well as shedding light on the role of SAT1 in p53-mediated tumor suppression.

Figure 3.1

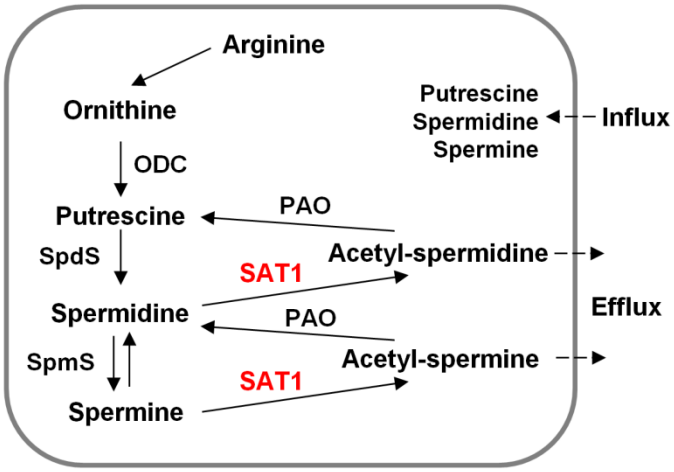

Figure 3.1. Polymaine metabolism pathway. SAT1, spermine/spermidine N^1-acetyltransferase 1; ODC, ornithine decarboxylase; PAO, N^1-acetylpolyamine oxidase; SpdS, spermidine synthase; SpmS: spermine synthase.

3.2 Materials and Methods

Cell culture and stable lines

All cells were cultured in 37°C incubator with 5% CO_2. All media used for cancer cell lines were supplemented with 10% fetal bovine serum (FBS), 100 units per ml penicillin and 100 μg/ml streptomycin (all Gibco). MEFs were generated from day 13.5 embryos according to standard procedures. FBS used for MEFs was heat-inactivated and supplemented with 1% non-essential amino acids. p53 inducible stable line was generated in H1299 cell line as previously described [81]. To induce the expression of p53, 0.5 μg/mL of tetracycline was added into culture medium. To generate *SAT1* inducible stable line, human *SAT1* complementary DNA was cloned into Tet-on pTRIPZ inducible expression vector (Thermo Open Biosystems) followed by transfection using Lipofectamine 2000 (Invitrogen), selection and maintenance with puromycin (1 μg/mL) in DMEM medium containing 10% tetracycline-free FBS. 0.5 μg/ml of doxycycline was used to induce the expression of SAT1. p53 CRISPR-cas9 knockout U2OS cell lines were generated by transfection of p53 double nickase plasmid (sc-416469-NIC, Santa Cruz) followed by selection with puromycin (1 μg/mL). Similarly, *SAT1* CRISPR-cas9 knockout U2OS cell lines were generated by transfection of pGL3-U6-sgRNA-PGK-puromycin vectors containing guide RNAs targeting exon 4, and pST1374-Cas9 vector. Guide RNA sequences targeting *SAT1* gene are 5'- GTCATAGGTAAAATAGTACATGG -'3 and 5'-

TGGCAAGTTATTGTATCTTGAGG -3'. Single colonies with p53 or *SAT1* knockout were selected and used for experiments. Knock-down of *SAT1* was performed by transfection of MEFs with siRNA duplex oligoset (On-Target-Plus Smartpool L05579601, Dharmacon) two times with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Western blotting and antibodies

Cell lysates were prepared in Flag lysis buffer with fresh protease inhibitor cocktail. Protein extracts were analyzed by western blotting according to standard protocols using primary antibodies specific for p53 (human: DO-1, Santa Cruz), MDM2 (Ab5, Millipore), TIGAR (E-2, Santa Cruz), PUMA (H-136, Santa Cruz), p21 (SX118, Santa Cruz), Actin (A3853, Sigma-Aldrich), SAT1 (H-7, Santa Cruz), Cleaved-caspase3 (Asp175, Cell Signaling Technologies), Cleaved-PARP(9542S, Cell Signaling Technologies) and GPX4 (ab125066, Abcam). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (GE Healthcare) were used and signals were detected on autoradiographic films with Pierce ECL western blotting detection system or SuperSignal West Dura reagents (Thermo scientific).

RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol Reagent (Life Technologies) according to manufacturer's protocol. cDNA was synthesized from total RNA using M-MulV Reverse

Transcriptase kit (NEB). PCR analysis was performed using Applied Biosystems 7500 Fast System. For the qRT-PCR analysis of human transcripts the following primers were used:

SAT1 forward 5’ -CCGTGGATTGGCAAGTTATT- 3’,

SAT1 reverse 5’ -TCCAACCCTCTTCACTGGAC-3’,

PTGS2 forward 5’ -CTTCACGCATCAGTTTTTCAAG- 3’,

PTGS2 reverse 5’ -TCACCGTAAATATGATTTAAGTCCAC- 3’,

ALOX15 forward 5’ -AGCCTGATGGGAAACTCTTG- 3’,

ALOX15 reverse 5’ -AGGTGGTGGGGATCCTGT -3’,

ALOX5 forward 5’ -CCTCAGGCTTCCCCAAGT- 3’,

ALOX5 reverse 5’ -GAAGATCACCACGGTCAGGT- 3’,

ALOX12 forward 5’ -GCTCCTGGAACTGCCTAGAA- 3’,

ALOX12 reverse 5’ -TCATCATCCTGCCAGCACT- 3’,

GPX4 forward 5’ -TTCCCGTGTAACCAGTTCG- 3’,

GPX4 reverse 5’ -CGGCGAACTCTTTGATCTCT- 3’,

GAPDH forward 5’ -ATCAATGGAAATCCCATCACCA- 3’,

GAPDH reverse 5’ -GACTCCACGACGTACTCAGCG- 3’.

For the qRT-PCR analysis of mouse transcripts the following primers were used:

SAT1 forward 5’ -GGCTAAATTTAAGATCCGTCCA- 3’,

SAT1 reverse 5’ -CATGTATTCATATTTAGCCAGTTCCTT- 3’.

Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed as previously described in H1299 cells transfected with empty vector or pCIN4-p53 [143]. Primers used for ChIP quantitative-PCR are:

SAT RE1 forward 5'- CAGTAGGGTTTCCGCCAAG -3',

SAT1 RE1 reverse 5’- AACCCGGAGGACAAAAGTG -3’,

SAT RE2 forward 5'- TCCTGAGTTTGCTTCCCACT -3',

SAT1 RE2 reverse 5’- GGTGTGTCCCCCAGTAACAT -3’,

SAT RE3 forward 5'- CACTGATTCTCAACTGCCAAA -3',

SAT1 RE3 reverse 5’- CAGAAGCAGAGGAGGAAAAGG -3’,

SAT RE4 forward 5'- CAAAAGACCACCCCTCACAT -3',

SAT1 RE4 reverse 5’- CCTAGGGCAGGAAGGGTAAC -3’,

TIGAR forward 5’- CGGCAGGTCTTAGATAGCTT -3’,

TIGAR reverse 5’- GGCAGCCGGCATCAAAAACA -3’.

Cell death count, drugs and inhibitors

Cells were trypsinized, collected and stained with trypan blue, followed by counting with a hemocymeter using standard protocol. Cells stained blue under the microscope were considered as dead cells. Nutlin (Sigma) was used in experiments at a concentration of 10μM.

DNA-damaging agent doxorubicin (Dox, Sigma) was used at 0.2 μg/mL. ROS generator tert-butyl hydroperoxide (TBH, Sigma) was used at a concentration of 60μM in H1299 cells, 350μM in U2OS cells and 150μM in MEFs respectively. Specific cell death inhibitors were used in the experiments with indicated concentrations as listed: Z-VAD-fmk (caspase 3 inhibitor, Sigma), 10 μg/mL; Necrostatin-1 (necroptosis inhibitor, Sigma), 10 μg/mL; Ferrostatin-1 (Ferroptosis inhibitor, Xcess Biosciences), 2 μM; 3-MA (autophagy inhibitor, Sigma), 2mM; and PD146176 (ALOX15 inhibitor, Sigma), 1 μM.

Analysis of reactive oxygen species (ROS) production

Cells were washed with PBS (Containing Ca^{2+} and Mg^{2+}) once, and then incubated with PBS containing 2μM C11-BODIPY(581/591) or 25μM H_2DCFDA (both from Invitrogen) at 37°C for 30min in tissue culture incubator. Cells were then washed, harvested by trypsinization and resuspended in 500μl fresh PBS. ROS levels were analyzed using a Becton Dickinson FACScalibur machine through FL1 channel, and data were analyzed using CellQuest. 10,000 cells were analyzed in each sample.

Mouse xenograft

SAT1 Tet-on stable H1299 cells were trypsinized, counted and then 1.5×10^6 cells were mixed with Matrigel (BD Biosciences) at 1:1 ratio (volume) and injected subcutaneously into nude mice (NU/NU, Charles River). Mice were fed either with control food or food containing

doxycycline hyclate (Harlem, 625 mg kg^{-1}). Four weeks after injection, mice were euthanized and tumors were dissected from under the skin. All procedures performed in this study are approved by the Institutional Animal Care and Use Committee at Columbia University.

3.3 Results and Discussion

3.3.1 *SAT1* is induced by p53

In normal cells, the p53 protein is controlled at extremely low levels by its negative regulator MDM2 [144]. Nutlin, a small-molecule antagonist of MDM2, inhibits the interaction between p53 and MDM2, and subsequently activates the transcription of p53 downstream targets [145]. To identify novel metabolic targets of p53, melanoma cell line A375 expressing wild-type p53 was either untreated or treated with Nutlin, and total RNA derived from these cells were subjected to RNA sequencing. In our previous section, we have identified *PHGDH* from the RNA sequencing result as a novel metabolic target of p53 that is critical for inducing the apoptotic response upon serine starvation [143]. In addition, we also identified mRNA levels of *SAT1* to be significantly up-regulated upon p53 activation (Figure 3.2A). It is well known that p53 can also be activated by DNA damage. To confirm that *SAT1* is regulated by p53, various human cancer cell lines MCF7, U2OS, A375 and H1299 were either untreated or treated with Nutlin or DNA- damage drug doxorubicin. As a result, *SAT1* mRNA levels were significantly up-regulated upon both Nutlin and doxorubicin treatment in cancer cell lines expressing wild-type p53 (U2OS, MCF7 and A375), whereas no apparent effects were detected in p53-null cell line H1299 (Figure 3.2B). Similarly, increase of *SAT1* mRNA levels upon Nutlin treatment and DNA damage were also observed in human renal cell carcinoma (RCC) cell lines expressing

wild-type p53 (HA251, HA212 and AU-48) (Figure 3.2C). However, expression of *SAT1* was not affected by both drugs in p53 mutant RCC cell lines (A704, SKRC-44 and SKRC-42) (Figure 3.2C).

To confirm that the transcription regulation of *SAT1* is dependent on p53, we generated p53-knockout U2OS cell line using CRISPR-cas9 technology. As shown in Figure 3.3A, p53 protein, as well as Nutlin-induced activation of downstream targets p21, TIGAR and MDM2, was completely abolished upon p53-knockout (p53 CRISPR). Notably, *SAT1* activation was also abrogated in p53-knockout U2OS cells treated with Nutlin (Figure 3.3B). Together, these data indicate that *SAT1* gene expression is enhanced in the presence of activated p53.

Figure 3.2

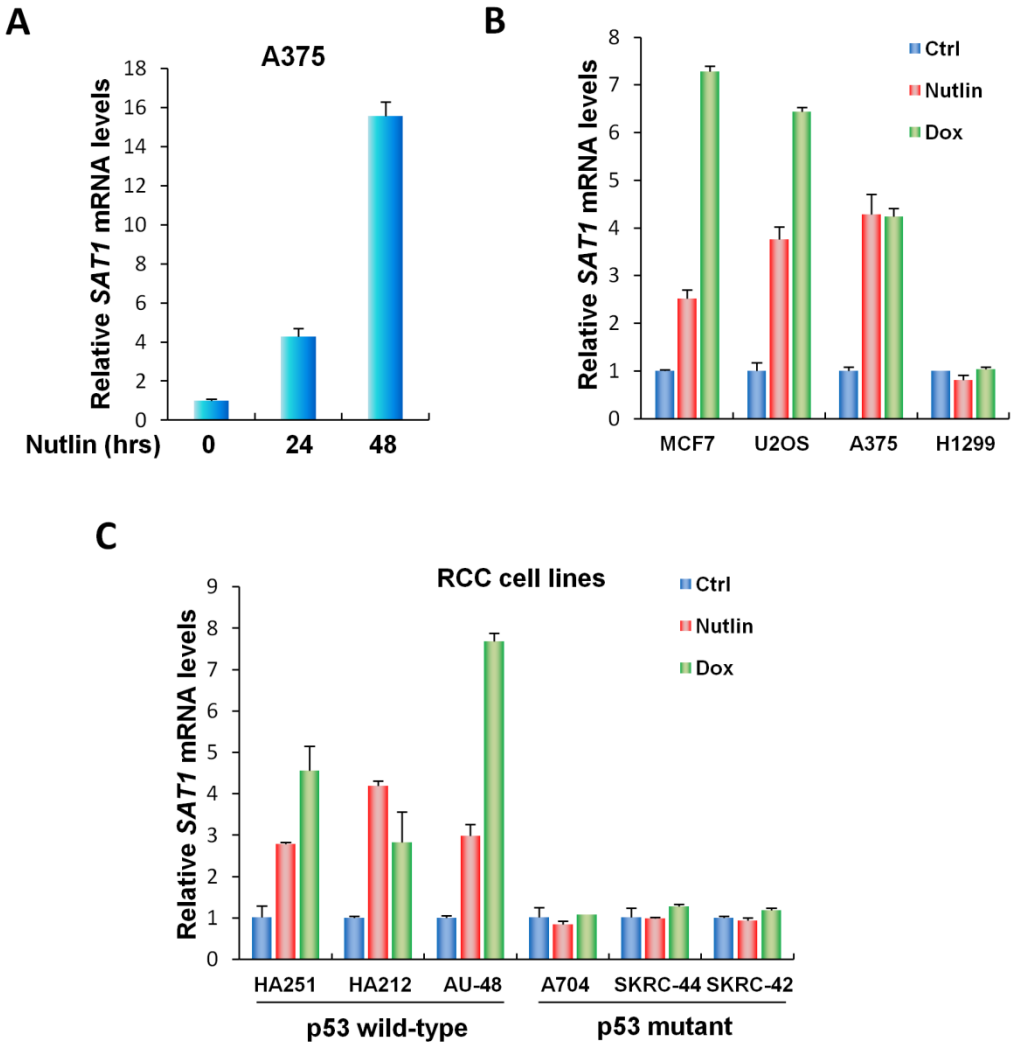

Figure 3.2. DNA damage and Nutlin induce p53-dependent activation of *SAT1*. (A) Quantitative RT-PCR (qRT-PCR) analysis of *SAT1* transcript level was performed with total RNAs purified from A375 cells treated with Nutlin (10μM) for the indicated times. **(B)** qRT-PCR analysis of the mRNA expression levels of *SAT1* in the indicated cancer cell lines (MCF7, U2OS, A375, H1299) untreated (Ctrl) or treated with Nutlin (10μM) or doxorubicin (Dox) (0.2μg/mL) for 24 hours. **(C)** Indicated Renal cell carcinoma cell lines were untreated or treated with Nutlin (10μM) or Dox (0.2μg/mL) for 24 hours, and *SAT1* mRNA levels were measured using qRT-PCR. All mRNA expression levels were normalized with GAPDH. Error bars represent s.d. from three experiments.

Figure 3.3

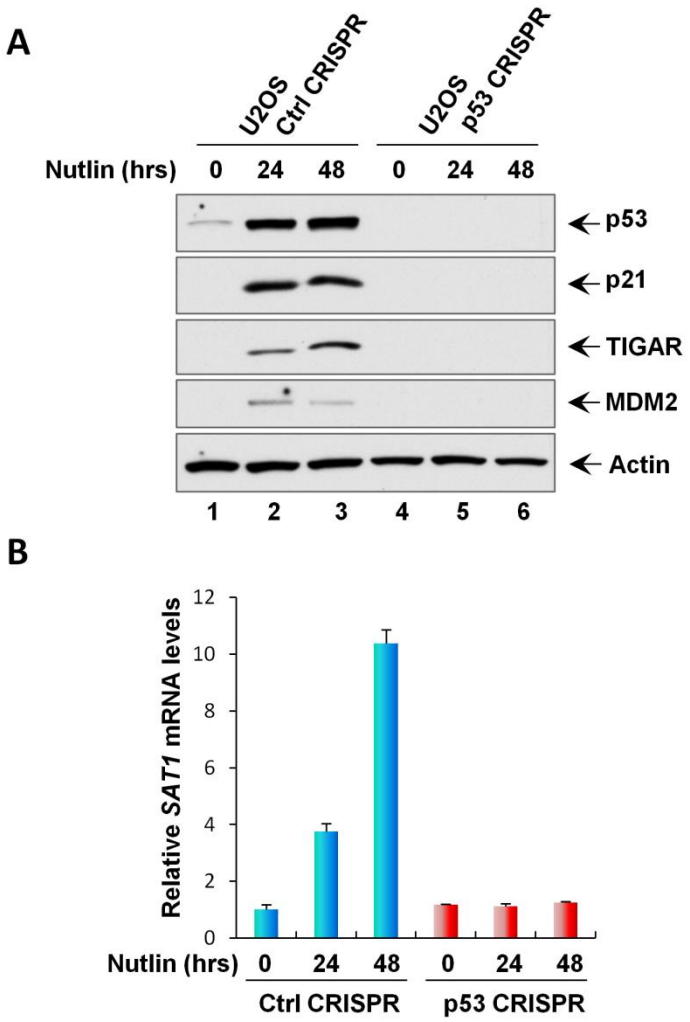

Figure 3.3. Nutlin-induced activation of *SAT1* is p53-dependent. (A) U2OS Ctrl CRISPR and p53 CRISPR cell lines were treated with Nutlin (10μM) for the indicated times, and total protein lysates were subjected to Western blotting analysis for the expression of p53, p21, TIGAR, MDM2 and Actin. **(B)** *SAT1* transcript levels were measured by qRT-PCR in U2OS Ctrl CRISPR and p53 CRISPR cell lines treated with Nutlin (10μM) for the indicated times. All mRNA expression levels were normalized with GAPDH. Error bars represent s.d. from three experiments.

3.3.2 Identification of *SAT1* as a p53 target

To further explore whether *SAT1* can be induced by exogenous p53, we established a Tet-on p53-inducible cell line in H1299 in which p53 expression is inducible by the addition of tetracycline. As expected, p53 was able to activate the expression of MDM2, TIGAR, PUMA (also known as BBC3) and p21 (also known as CDKN1A) (Figure 3.4A). Notably, *SAT1* mRNA levels were also up-regulated at various time points after p53 induction (Figure 3.4B).

The promoter region of the human *SAT1* gene at chromosome Xp22.1 contains two potential sites that match the consensus p53-binding sequence (Figure 3.5A). Chromatin immunoprecipitation (ChIP) analysis of H1299 cells expressing exogenous p53 revealed increased binding of p53 with two indicated binding sites (Figure 3.5B). Moreover, overexpression of wild-type p53 by transient transfection induced the mRNA levels of *SAT1*, whereas three p53 hot-spot mutants (R175H, R273H and R248W) did not affect the expression of *SAT1* (Figure 3.5C). Collectively, these data demonstrate that *SAT1* is a novel transcriptional target of p53.

Figure 3.4

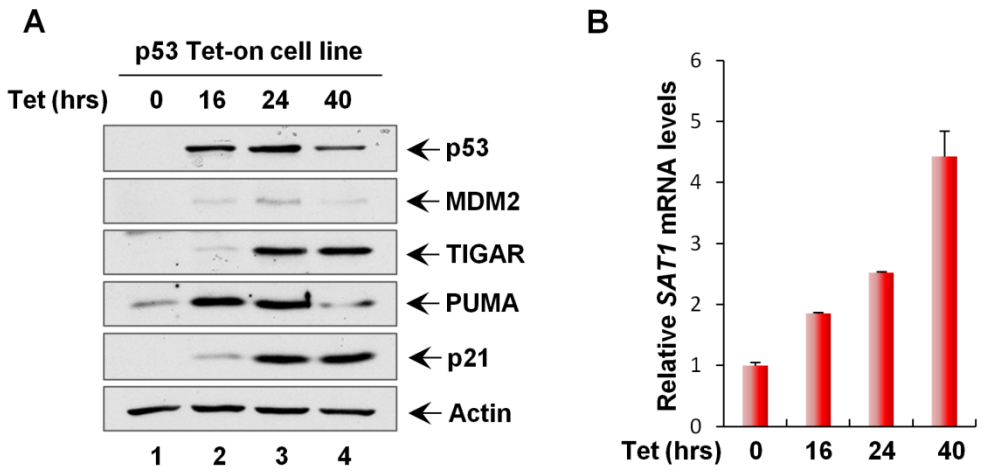

Figure 3.4. p53 induces the transcription of *SAT1*. (A) p53 Tet-on H1299 cells were induced with 0.5μg/mL tetracycline (Tet) for indicated times, and total protein lysates were analyzed by Western blotting using antibodies against p53, MDM2, TIGAR, PUMA, p21 and Actin. **(B)** *SAT1* mRNA expression levels were measured by qRT-PCR in p53 Tet-on H1299 cells induced with 0.5μg/mL tetracycline for the indicated times.

Figure 3.5

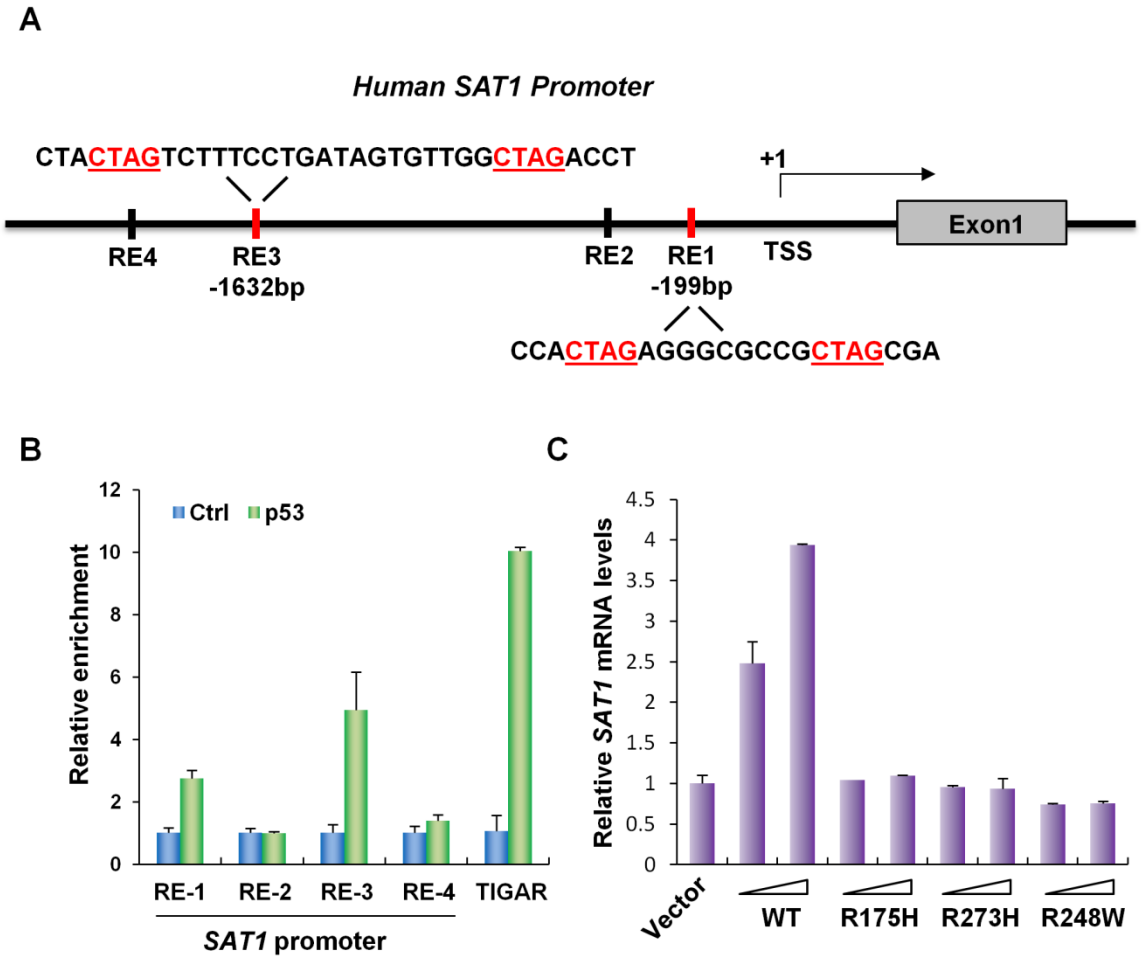

Figure 3.5. *SAT1* is a transcriptional target of p53. (A) Schematic representation of the promoter region in human *SAT1* gene. The p53 binding sites upstream of the first exon are indicated as responsive elements (RE). TSS, transcription start site. **(B)** ChIP-qPCR was performed in H1299 cells transfected with empty vector (Ctrl) or p53. **(C)** H1299 cells were transfected with empty vector or increasing amount of p53 wild-type or mutant vectors (R175H, R273H and R248W), and *SAT1* mRNA levels were analyzed by qRT-PCR.

3.3.3 Effect of SAT1 overexpression on growth arrest, apoptosis and tumorigenesis

SAT1 is a key polyamine catabolism enzyme that mediates the acetylation of spermidine and spermine. Overexpression of SAT1 has been implicated to cause a rapid depletion of polyamine pool [138]. To investigate the effect of SAT1 on cell proliferation and survival in a physiological setting, we generated a *SAT1* tetracycline-inducible (Tet-on) cell line using p53-null H1299 cells. Upon addition of tetracycline, both SAT1 protein and mRNA levels were increased in a time-dependent manner (Figure 3.6A and 3.6B). Surprisingly, no obvious growth arrest or cell death was observed upon SAT1 induction (Figure 3.6C). In addition, expression of apoptosis markers (PUMA, cleaved-caspase3 and cleaved-PARP) were not detected in cell lysates from Tet-on cells expressing SAT1, indicating the absence of apoptosis (Figure 3.6D).

To explore whether SAT1 has tumor suppressive activities *in vivo*, we injected the *SAT1* Tet-on H1299 cells into nude mice, and fed the mice with tetracycline-containing food to induce the expression of SAT1 in xenograft cells. Upon SAT1 induction, the growth of p53-null H1299 cells was dramatically reduced in the xenograft tumor growth assay (Figure 3.7A and 3.7B). Notably, the expression data from Oncomine database revealed that *SAT1* expression is down-regulated in a variety of human cancers, including invasive breast carcinoma, lung carcinoid tumor, B-cell acute lymphoblastic leukemia and myxoid/round cell liposarcoma (Figure 3.8). Further analysis of 21 pairs of human tumors versus adjacent normal tissues

obtained from our local tumor bank also showed that *SAT1* is down-regulated in 86% of the human cancer specimens (6/6 kidney tumor samples, 5/5 breast tumor samples, and 7/10 colon tumor samples) (Figure 3.9). These data implicate that SAT1 has tumor suppressive activities independent of cell growth arrest and apoptosis, and that it may be a common oncogenic target during tumorigenesis in various human malignancies.

Figure 3.6

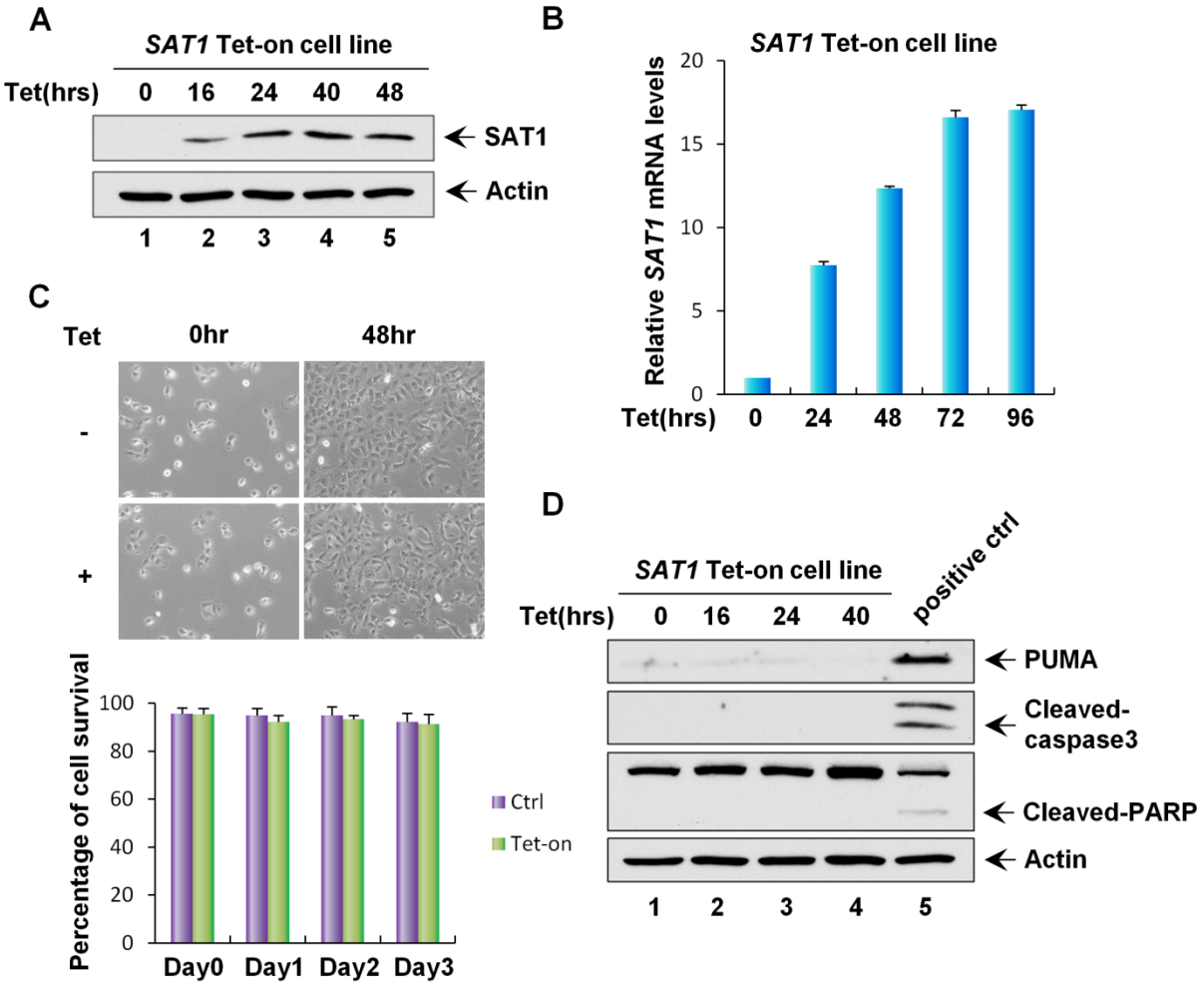

Figure 3.6. SAT1 overexpression does not induce growth arrest and apoptosis. (A) *SAT1* Tet-on inducible stable line cells were treated with 0.5μg/mL tetracycline for indicated times followed by western blot analysis. Actin was used as a loading control. **(B)** qRT-PCR analysis of mRNA levels of *SAT1* in *SAT1* Tet-on stable line cells at the indicated times after induction. **(C)** Representative phase-contrast images of *SAT1* Tet-on stable cells un-induced or induced with 0.5μg/mL tetracycline for 48 hours (magnification, ×10). Percentage of cell survival at the indicated times is shown as mean ± s.d. **(D)** *SAT1* Tet-on stable cells were induced with 0.5μg/mL tetracycline for indicated times, and total protein lysates were subjected to western blot analysis for the expression of PUMA, Cleaved-caspase3, Cleaved-PARP and Actin.

Figure 3.7

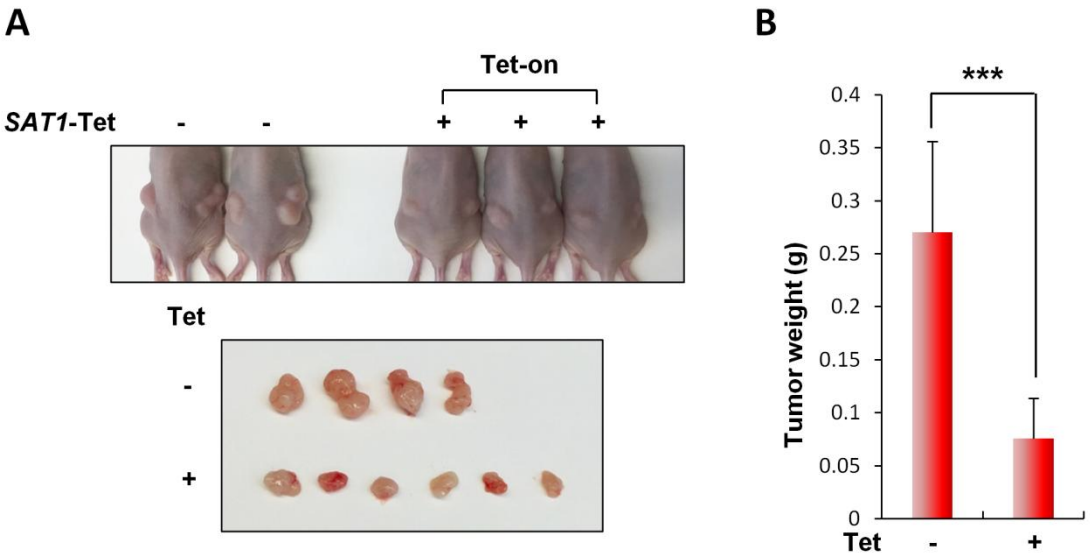

Figure 3.7. SAT1 suppresses xenograft tumor growth *in vivo.* (A) Xenograft tumors from *SAT1* Tet-on cells. **(B)** Tumor weight was determined (error bars, s.d. from four tumors in control and six tumors in tet-on). *** is used to indicate statistical significance corresponding to *P* value < 0.001.

Figure 3.8

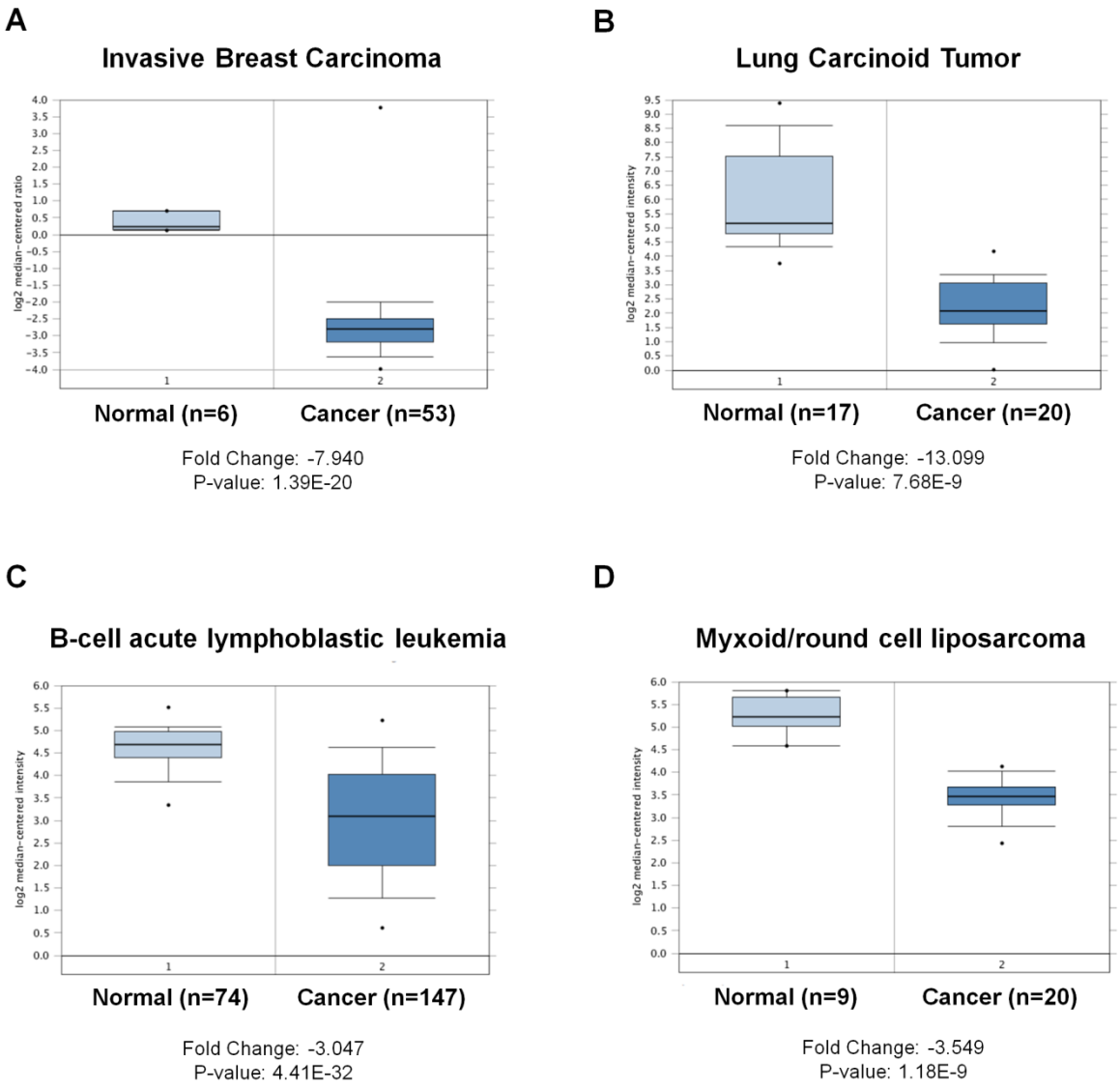

Figure 3.8. *SAT1* is down-regulated in human cancers. (A, B, C and D) Data-sets obtained from Oncomine database showing that the transcription levels of *SAT1* are lower in tumors (cancer) than in their adjacent normal tissues (normal) in indicated cancer types. The actual fold change and p-value are indicated on the bottom of each panel.

Figure 3.9

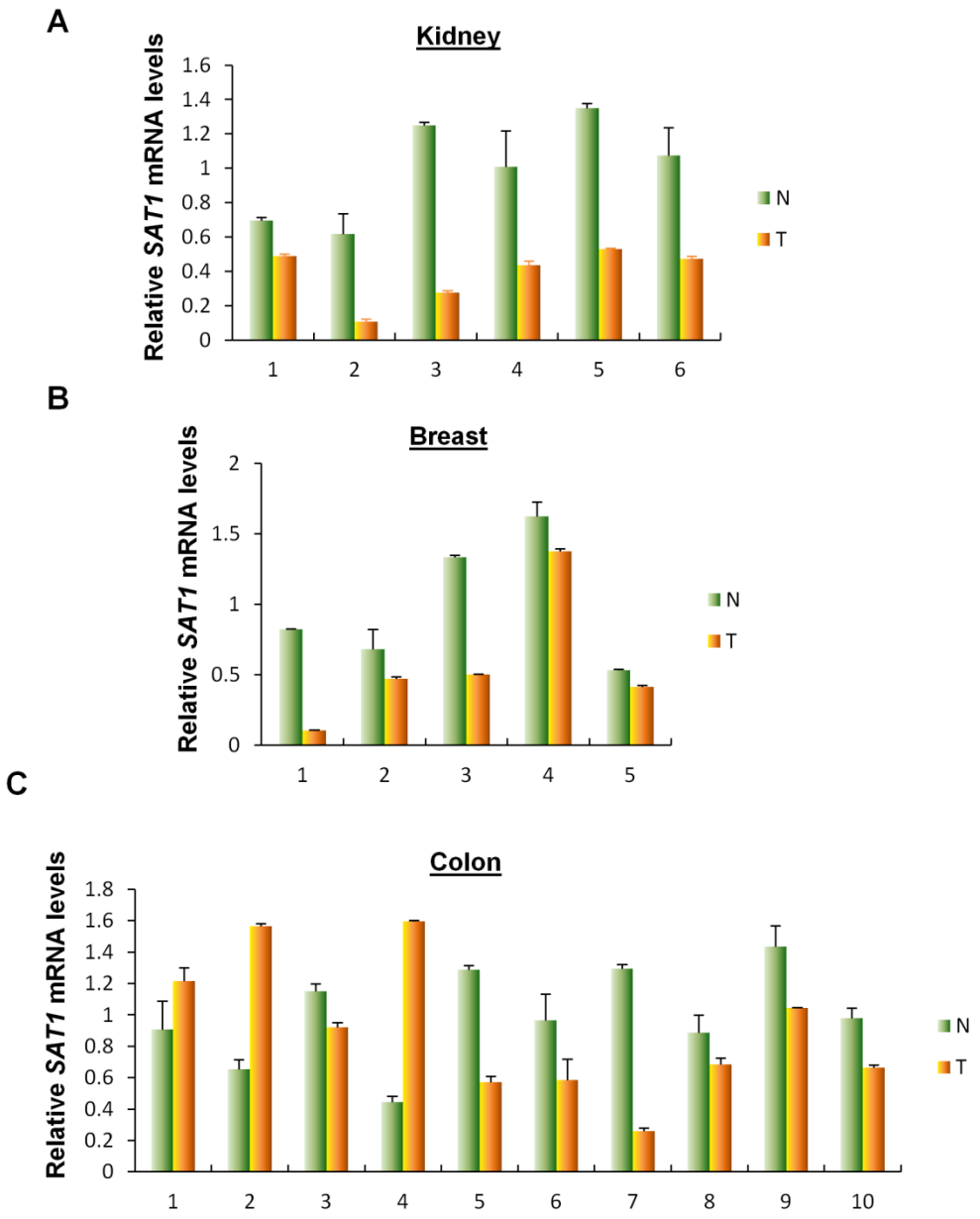

Figure 3.9. *SAT1* is down-regulated in tumors of human cancer patients. (A, B and C) Quantitative RT-PCR analysis of the expression levels of *SAT1* in paired normal and cancer tissues from kidney (A), breast (B) and colon (C). Average expression levels from normal tissues were normalized to 1 in each type of cancer. Data shown in mean ± s.d. from two technical replicates. N, normal tissue; T, cancer tissue.

3.3.4 SAT1 overexpression leads to lipid peroxidation and ferroptosis upon ROS stress.

Our previous studies have revealed that tumor suppression mediated by p53 can occur in the absence of growth arrest, apoptosis and senescence [97]. Notably, p53-mediated ferroptosis in response to ROS stress through suppressing SLC7A11 is a novel tumor suppression mechanism that has been discovered recently [81]. In fact, polyamine metabolism has been implicated to be involved in ROS stress response, as the natural polyamine spermine can function as a free radical scavenger, while catabolization of polyamine by SAT1 and polyamine oxidase (PAO) gives rise to H_2O_2 and increase oxidative stress [146-148]. Nevertheless, the cell death response upon SAT1 expression in the setting of oxidative stress exposure is unexplored. To evaluate whether SAT1 overexpression modulates ROS stress response, we treated *SAT1* Tet-on cells with ROS inducing agent tert-butyl hydroperoxide (TBH) as previously described [149]. As shown in Figure 3.10, no obvious cell death was observed upon either SAT1 induction or ROS treatment alone. However, the combination of SAT1 induction and ROS treatment induced significant cell death (Figure 3.10). The mode of cell death was then confirmed by treatment of different cell death inhibitors. Notably, Ferrostatin-1 (Fer-1), a specific ferroptosis inhibitor, completely rescued SAT1 and ROS-induced cell death. In contrast, inhibitors of apoptosis (Z-VAD-fmk), necroptosis (necrostatin-1) and autophagy (3-methylademine) failed to suppress cell death (Figure 3.10). This demonstrated that SAT1 expression triggers ferroptosis upon ROS stress.

Notably, the up-regulation of *Ptgs2* has recently been identified to be a potential molecular marker of ferroptosis by Stockwell's lab [150]. We therefore examined *Ptgs2* levels in xenograft tumors. Indeed, *Ptgs2* was found to be significantly up-regulated in the tumors when *SAT1* was induced, suggesting that ferroptosis is involved in tumor suppression (Figure 3.11).

Previous studies have indicated that lipid peroxidation is a crucial event on cell membrane that leads to ferroptosis [84]. We then examined lipid ROS levels in *SAT1* Tet-on cells upon SAT1 induction and ROS treatment. As a result, SAT1 induction alone only had modest effect on lipid ROS, and ROS treatment alone elevated lipid ROS level by 4-fold (Figure 3.12A and 3.12B). However, the lipid ROS level was increased by 9-fold upon concomitant SAT1 induction and ROS treatment (Figure 3.12A and 3.12B). In contrast, no differences in cellular ROS levels were observed between cells with ROS treatment alone and those with the addition of SAT1 induction (Figure 3.12C and 3.12D). Taken together, these data demonstrate that SAT1 overexpression leads to lipid peroxidation and ferroptosis upon ROS stress.

Figure 3.10

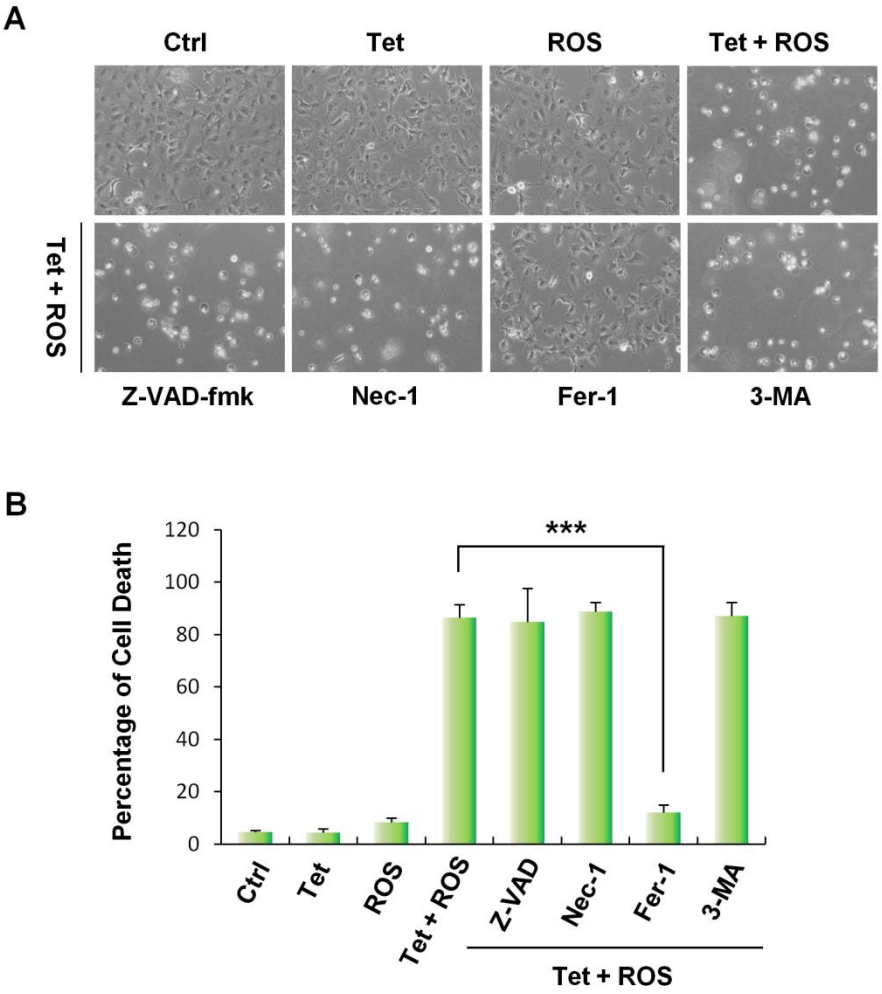

Figure 3.10. SAT1 overexpression leads to ferroptosis upon ROS stress. (A) Representative phase-contrast images of *SAT1* Tet-on cells treated with 0.5μg/mL tetracycline and 60μM TBH for 24 hours. The images also show cells treated with tetracycline and TBH with the addition of specific cell death inhibitors for 24 hours. Z-VAD-fmk, a caspase inhibitor, 10μg/mL; Necrostatin 1 (Nec-1), a necroptosis inhibitor, 10μg/mL; Ferrostatin-1 (Fer-1), a ferroptosis inhibitor, 2μM; 3-methyladenine (3-MA), an autophagy inhibitor, 2mM. **(B)** The percentages of cell death for all experiments shown in Figure 3.10A were measured by trypan blue exclusion assay.

Figure 3.11

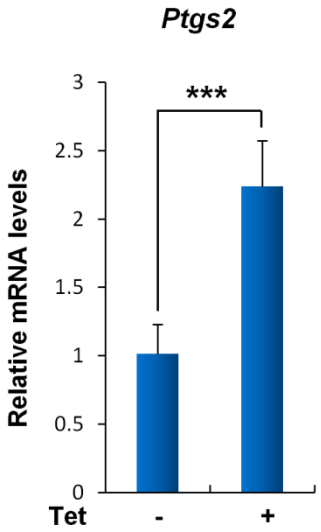

Figure 3.11. Ferroptosis marker *Ptgs2* is up-regulated in xenograft tumors with SAT1 induction. The mRNA levels of *Ptgs2* was determined by qRT-PCR analysis in 4 xenograft tumors without SAT1 induction and 6 xenograft tumors with SAT1 induction. Data is shown as mean ± s.d. *** is used to indicate statistical significance corresponding to *P* value < 0.001.

Figure 3.12

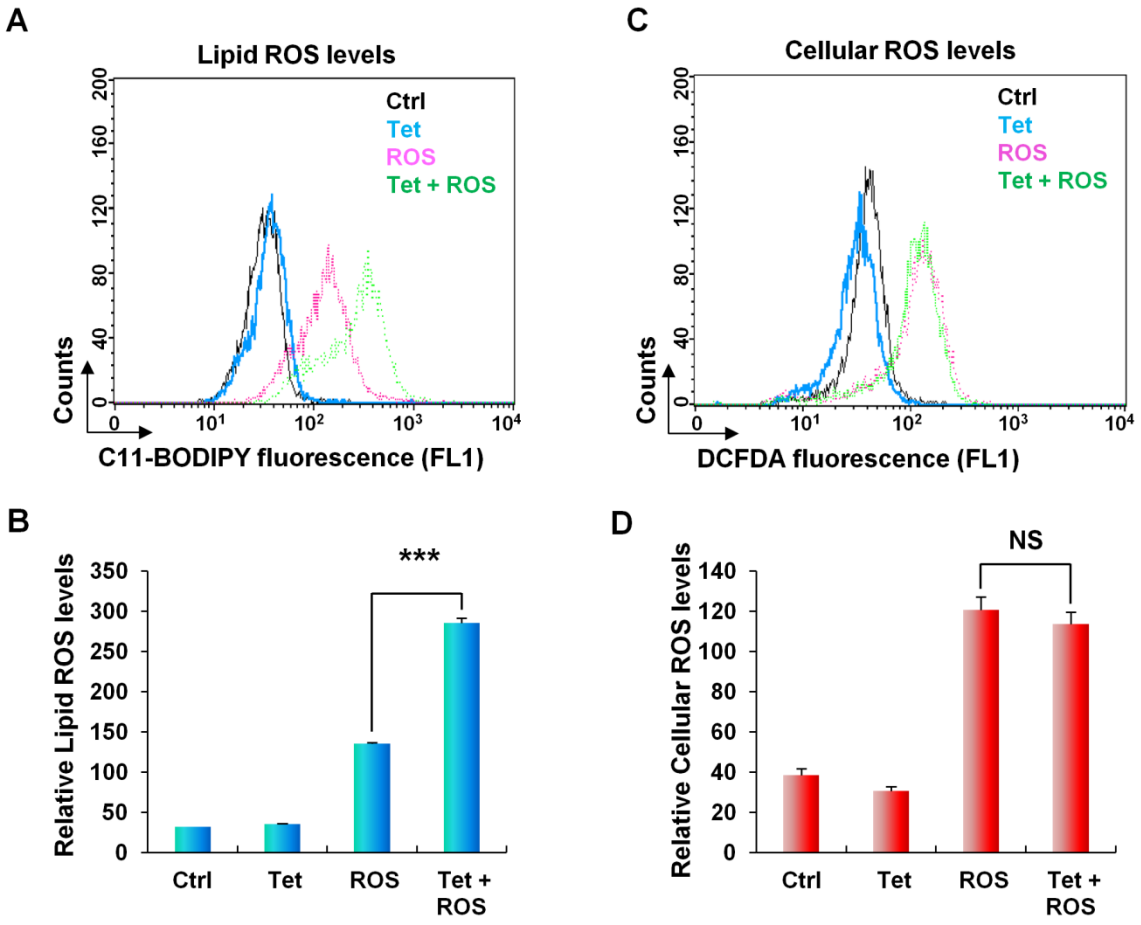

Figure 3.12. SAT1 overexpression increases lipid peroxidation upon ROS stress. (A and C) Lipid and cytosolic ROS production in *SAT1* Tet-on cells treated with tetracycline and TBH for 24 hours were assessed by flow cytometry using C11-BODIPY and H_2DCFDA. **(B and D)** Quantification of lipid and cytosolic ROS levels from three representative experiments are shown (mean ± s.d.) *** is used to indicate statistical significance corresponding to *P* value < 0.001. NS indicates no significant difference.

3.3.5 SAT1 contributes to p53-mediated ferroptosis upon ROS stress

Our results that *SAT1* is a novel transcriptional target of p53 implicate that SAT1 may contribute to p53-mediated ferroptotic cell death and ROS response. To test our hypothesis, we established a *SAT1*-knockout CRISPR-cas9 cell line in p53 wild-type U2OS cells. Because the SAT1 antibody could not detect endogenous SAT1 protein, we designed a qRT-PCR primer in between the targeting regions of two guide RNAs, therefore were able to confirm knockout efficiency through qRT-PCR. As shown in Figure 3.13A, the mRNA levels of *SAT1* were markedly increased when p53 is activated by Nutlin in mock-knockout U2OS cells (Ctrl CRISPR). In contrast, *SAT1* expression was undetectable with or without Nutlin treatment in *SAT1*-knockout U2OS cells (*SAT1* CRISPR) (Figure 3.13A). Notably, *SAT1* deficiency did not affect p53-mediated growth arrest and apoptosis, as the expression of p21 and PUMA in response to the Nutlin treatment were not changed upon *SAT1*-knockout (Figure 3.13B). To evaluate the role of SAT1 in p53-mediated ferroptosis, U2OS mock-knockout and *SAT1*-knockout cells were treated with Nutlin and ROS. Consistent with our previous results, Nutlin or ROS treatment alone failed to elicit a cell death response, whereas combination treatment with both Nutlin and ROS induced massive ferroptotic cell death in mock-knockout U2OS cells (Figure 3.14). Notably, the cell death was significantly abrogated upon knockout of *SAT1*, indicating that p53-mediated activation of SAT1 contributes to ferroptotic cell death in the

presence of ROS stress (Figure 3.14).

Figure 3.13

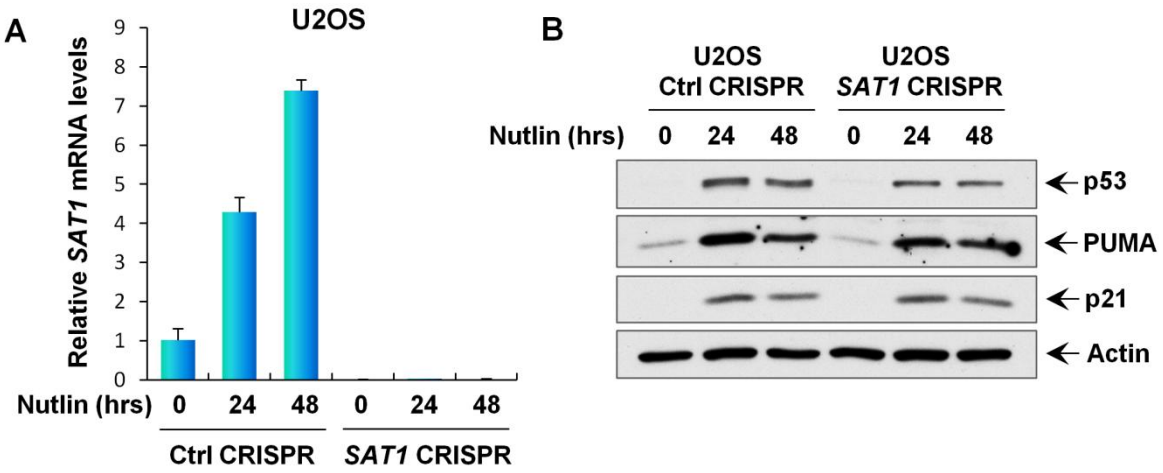

Figure 3.13. *SAT1* knockout does not affect p53-mediated cell cycle arrest and apoptosis. (A) qRT-PCR analysis of *SAT1* mRNA levels in U2OS control (Ctrl) CRISPR and *SAT1* CRISPR stable cell lines treated with 10μM Nutlin for indicated times. **(B)** U2OS control CRISPR and *SAT1* CRISPR stable cell lines were treated with 10μM Nutlin for indicated times, and total protein lysates were subjected to western blot analysis for the expression of p53, PUMA, p21 and Actin.

Figure 3.14

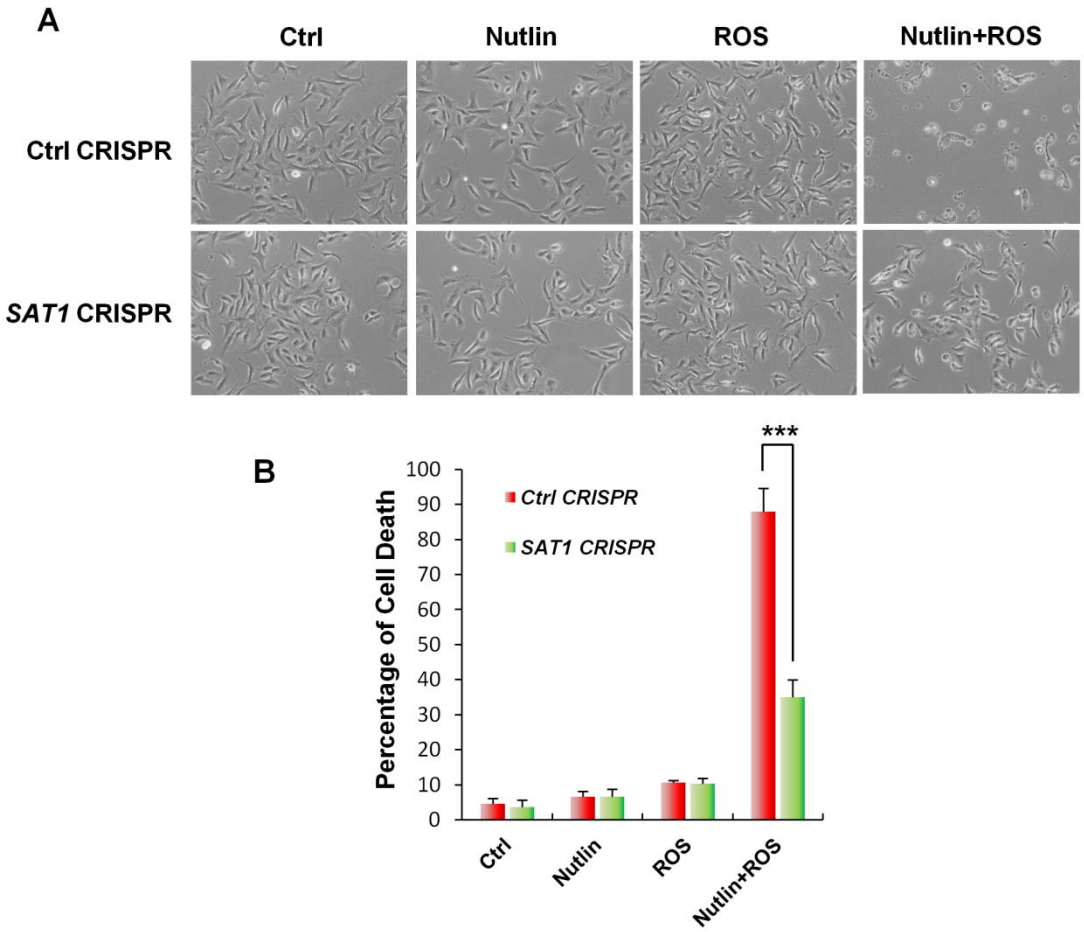

Figure 3.14. SAT1 contributes to p53-mediated ferroptosis upon ROS stress. (A) U2OS control CRISPR and *SAT1* CRISPR cells were treated with 10μM Nutlin and 350μM TBH for 24 hours when images were taken. **(B)** Quantification of cell death in Figure 3.14A from three technical triplicates (mean ± s.d.) is shown. *** is used to indicate statistical significance corresponding to *P* value < 0.001.

Previously, a p53 acetylation-deficient mutant $p53^{3KR}$(K117/161/162R) was found to retain the ability to promote ferroptosis [81]. Moreover, mice that harbor these mutations still retain intact tumor suppression [97]. Therefore, we examined the levels of *Sat1* transcripts in mouse embryonic fibroblasts (MEFs) derived from $p53^{+/+}$, $p53^{3KR/3KR}$ and $p53^{-/-}$ mice. qRT-PCR analysis revealed that *Sat1* expression is markedly increased in both $p53^{+/+}$ and $p53^{3KR/3KR}$ MEFs in response to Nutlin treatment, whereas no change was observed in $p53^{-/-}$ MEFs (Figure 3.15A). In addition, knock-down of *Sat1* by siRNA in $p53^{3KR/3KR}$ MEFs partially rescued ROS-induced ferroptosis, suggesting that SAT1 contributes, at least in part, to $p53^{3KR}$-mediated ferroptotic responses (Figure 3.15B-D). Collectively, these data implicate that p53-mediated regulation of SAT1 contributes to p53-mediated ferroptosis, ROS response and tumor suppression.

Figure 3.15

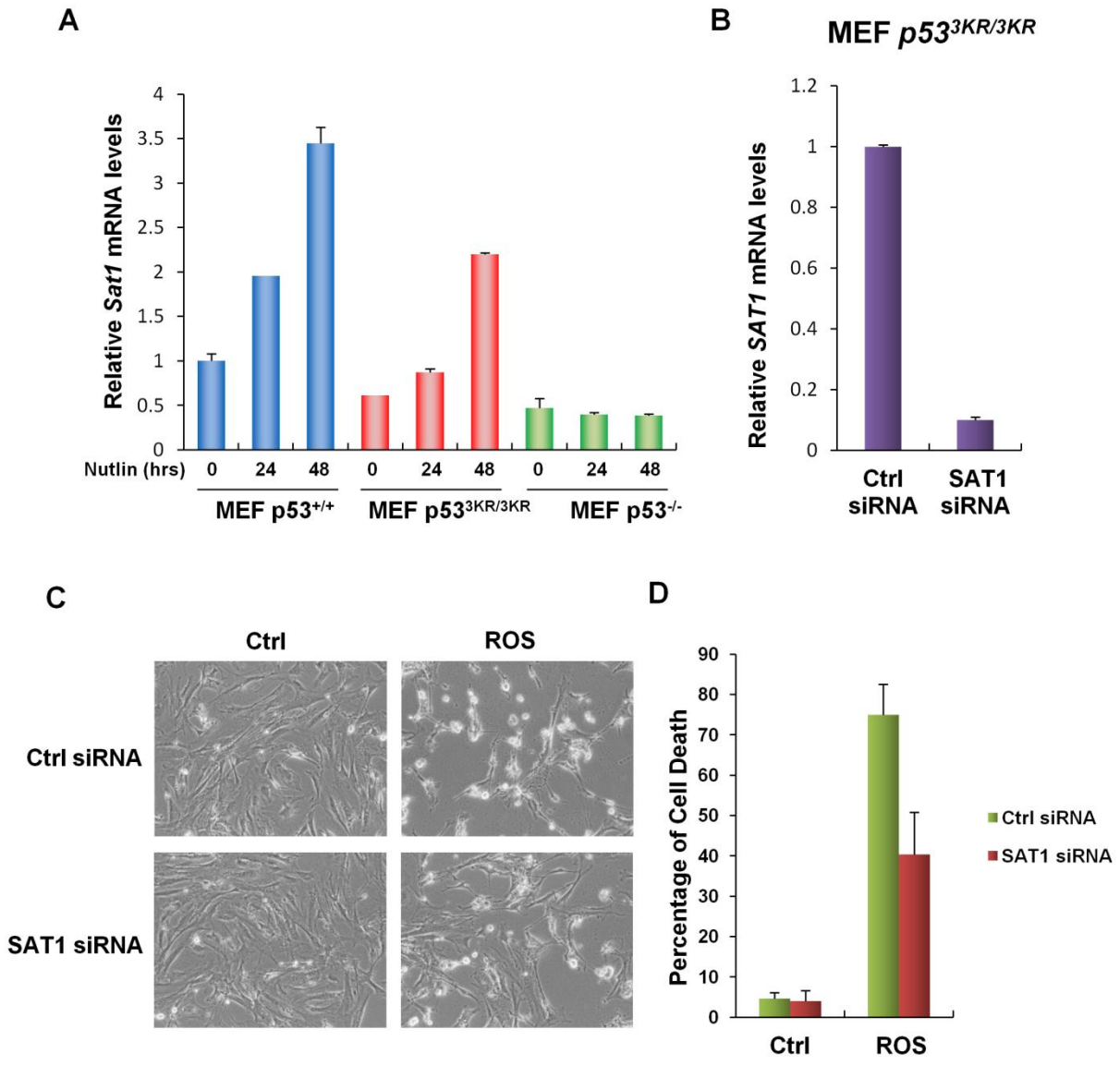

Figure 3.15. SAT1 contributes to p53-3KR-mediated ferroptosis upon oxidative stress. (A) MEFs from indicated genotype were treated with 10μM Nutlin, and *Sat1* transcript levels were measured by qRT-PCR. **(B)** qRT-PCR analysis of *SAT1* mRNA levels in $p53^{3KR/3KR}$ MEFs transfected with control siRNA or SAT1 siRNA for 48 hours. **(C)** $p53^{3KR/3KR}$ MEFs were transfected with control siRNA or SAT1 siRNA for 48 hours, and then treated with TBH (150μM) for 24 hours before images were taken. **(D)** Quantification of cell death in Figure 3.15C from three technical triplicates (mean ± s.d.) is shown.

3.3.6 Molecular mechanism of SAT1-induced ferroptosis

Although the mechanism of SAT1-induced ferroptosis upon ROS stress is yet unknown, there were some studies implicating that polyamine metabolism pathway could regulate histone modification, and therefore, alter gene expression [151, 152]. We hypothesized that SAT1 regulates ferroptosis through modulating the expression of components in the ferroptosis pathways. GPX4 is a glutathione peroxidase that functions as a central regulator of ferroptosis. Inhibition of GPX4 has been implicated to increase the level of lipid peroxidation and lead to ferroptosis [150]. Therefore, we examined GPX4 expression levels in *SAT1* Tet-on cells. However, no changes were observed at both the protein and mRNA levels of GPX4 upon SAT1 induction and ROS treatment (Figure 3.16). Our previous studies have also identified p53-mediated transcriptional repression of SLC7A11, a component of the cystine/glutamate antiporter found to be critical for ROS-induced ferroptosis [81]. Again, overexpression of SLC7A11 did not rescue SAT1-induced ferroptosis upon ROS stress, indicating that SAT1 functions independently or downstream of SLC7A11 (Figure 3.17).

Figure 3.16

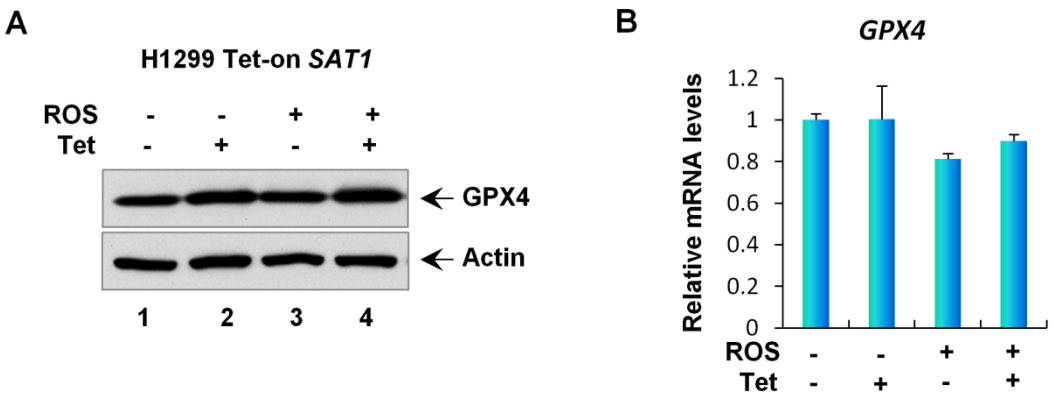

Figure 3.16. SAT1 does not affect GPX4 expression. (A) *SAT1* Tet-on cells were treated with tetracycline and TBH, and total cell lysates were subjected to western blot analysis for the expression of GPX4. Actin was used as a loading control. **(B)** qRT-PCR analysis of *GPX4* mRNA levels in *SAT1* Tet-on cells treated with tetracycline and TBH.

Figure 3.17

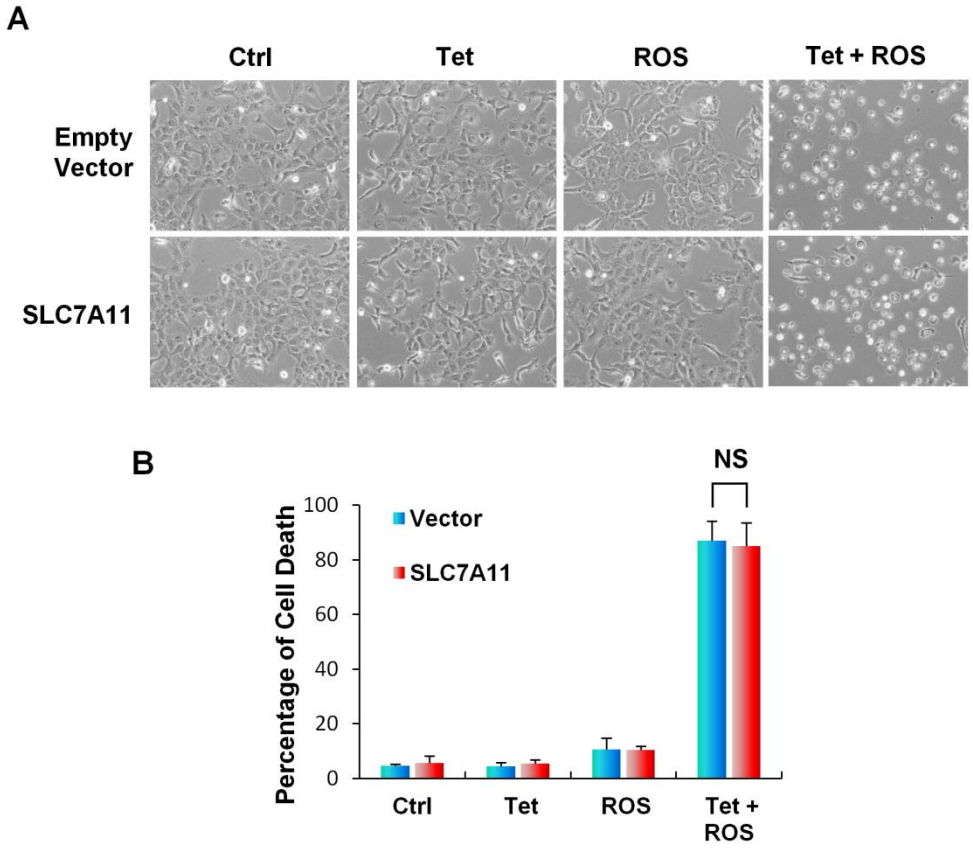

Figure 3.17. SLC7A11 overexpression does not rescue SAT1-induced ferroptosis upon ROS. **(A)** Representative phase-contrast images of *SAT1* Tet-on cells transfected with empty vector or vector expressing SLC7A11 followed by treatment of tetracycline and TBH (60μM) for 24 hours. **(B)** Quantification of cell death is shown in mean ± s.d. from three technical triplicates. NS indicates no significant difference.

Notably, lipid peroxidation is a critical event on cell membrane that leads to ferroptosis, and studies have revealed that arachidonate 15-lipoxgenase (ALOX15) is specifically responsible for oxidative stress-induced cell death among lipoxygenase family [153]. Interestingly, *ALOX15* expression levels were elevated upon SAT1 induction or with combination of ROS treatment, whereas no increase in levels were observed in the other two lipoxygenases (ALOX5, 5-lipoxygenase and ALOX12, 12-lipoxygenase) (Figure 3.18). In addition, SAT1 and ROS-induced ferroptosis was completely abrogated by PD146176, an ALOX15-specific inhibitor [154] (Figure 3.19A and 3.19B). Furthermore, induction of *ALOX15* expression level was also partially attenuated upon *SAT1* knockout after p53 activation via Nutlin, suggesting that ALOX15 is a downstream effector of p53-induced SAT1 (Figure 3.19C). Taken together, these data implicate that ALOX15 is critical for SAT1-induced ferroptosis upon ROS stress.

Figure 3.18

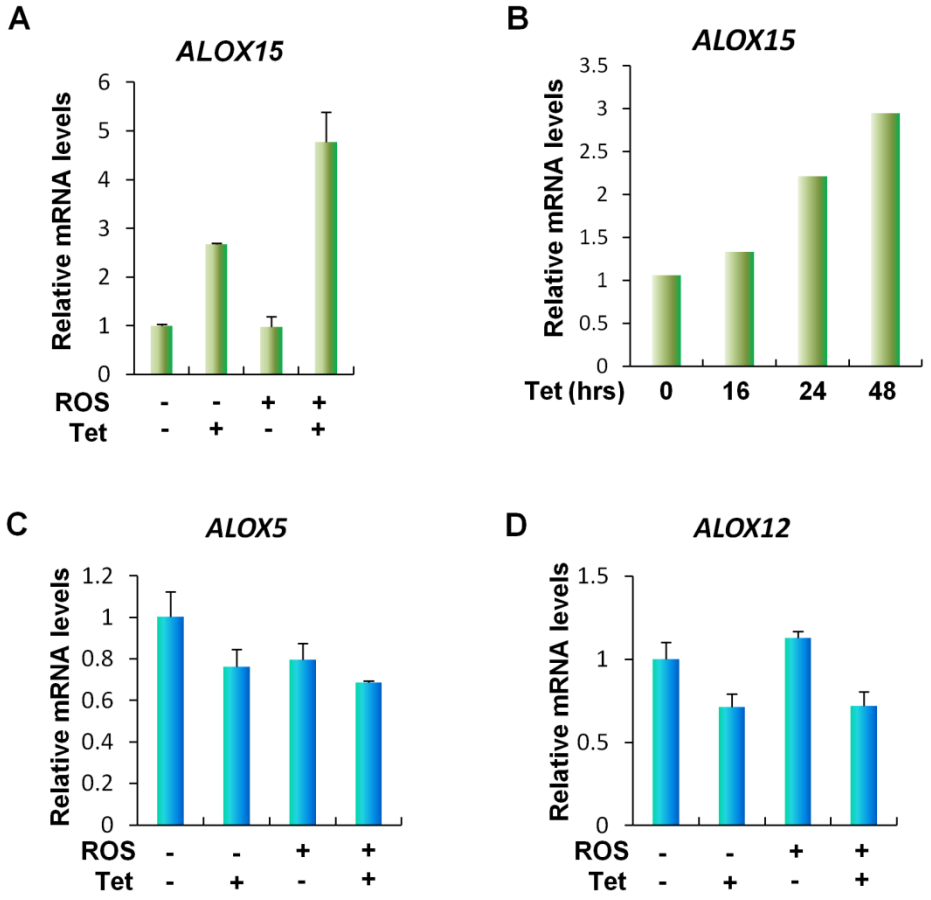

Figure 3.18. SAT1 induces the expression of ALOX15. (A) qRT-PCR analysis of *ALOX15* mRNA levels in *SAT1* Tet-on cells treated with tetracycline and TBH. **(B)** *SAT1* Tet-on cells were induced with 0.5µg/mL tetracycline for the indicated times, and *ALOX15* mRNA levels were examined using qRT-PCR. **(C and D)** qRT-PCR analysis of *ALOX5* and *ALOX12* mRNA levels in *SAT1* Tet-on cells treated with tetracycline and TBH.

Figure 3.19

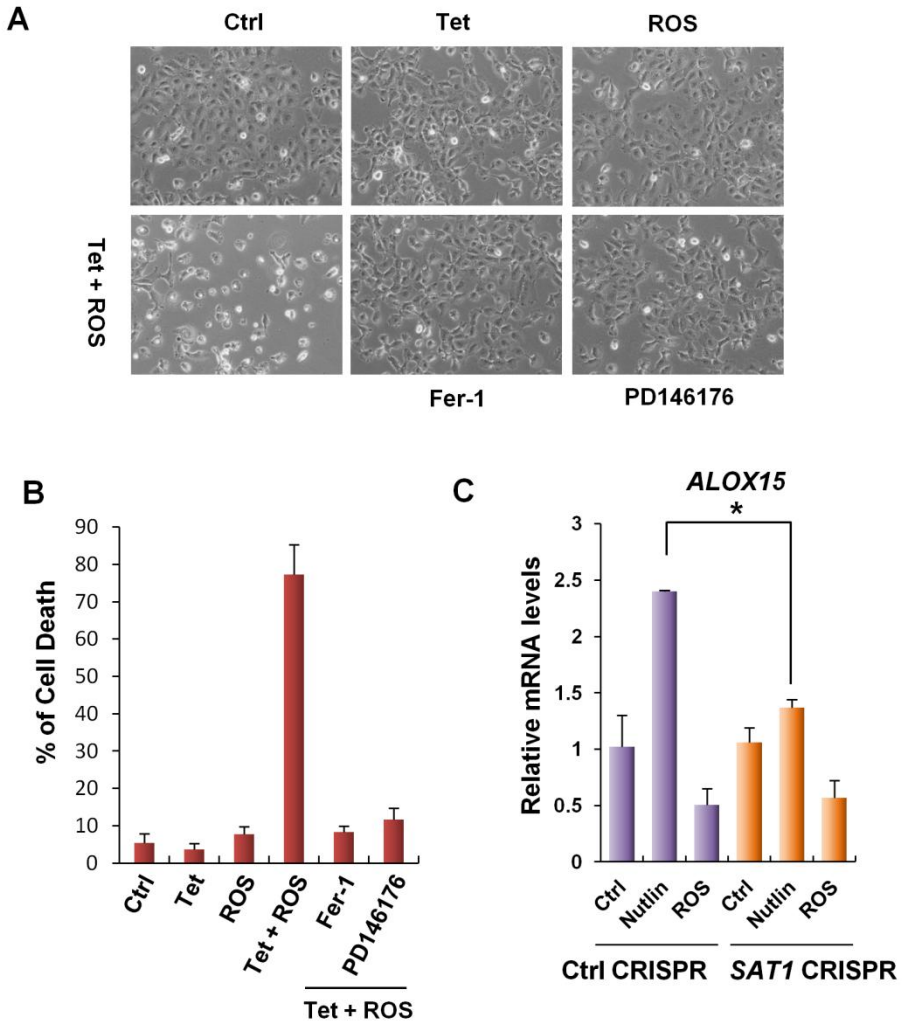

Figure 3.19. ALOX15 is a mediator during SAT1-induced ferroptosis upon ROS. (A) Representative phase-contrast images of *SAT1* Tet-on cells treated with tetracycline and TBH with the addition of ferroptosis inhibitor (Fer-1) or ALOX15 inhibitor (PD146176). **(B)** The percentages of cell death were measured by trypan blue exclusion assay. **(C)** U2OS control CRISPR and *SAT1* CRISPR cells were treated with 10μM Nutlin or 350μM TBH for 24 hours, and total RNA was extracted for the analysis of *ALOX15* mRNA levels using qRT-PCR. Data is shown in mean ± s.d. from three technical triplicates. * is used to indicate statistical significance corresponding to *P* value < 0.05.

CHAPTER 4

Dissecting the role of acetylation

in p53-mediated differential gene regulation and tumor suppression

4.1 Background and Rationale

Our studies on PHGDH and SAT1 led us to the question of how p53 differentially regulates a diverse array of downstream targets, and which functions of p53 contribute to p53-mediate tumor suppression. It is well-known that p53 activity is regulated by a complex network of fine-tuning mechanisms that include p53 stabilization, co-activator and repressor recruitment, and varieties of post-translational modifications, including ubiquitination, phosphorylation, acetylation, sumoylation, methylation, and neddylation [16]. Specifically, acetylation of p53 is indispensible for p53 activation, and is demonstrated to play a major role in differential regulation of downstream targets during stress responses [155]. Previous studies have revealed that acetylation at K120 by Tip60/MOF is critical for p53-mediated apoptotic responses [156, 157], while acetylation at K164 by CBP/p300, along with K120 acetylation, contributes to both p53-mediated cell cycle arrest and apoptosis [155]. More recently, we generated $p53^{3KR/3KR}$ knock-in mice in which acetylation at K117, K161, and K162 in mouse p53 (homologous to K120 and K164 in human p53) were abrogated by replacing the lysine residues with arginine. As a result, $p53^{3KR/3KR}$ mice completely lost p53-induced growth arrest, apoptosis, and senescence functions in response to cellular stresses [97]. Surprisingly, unlike p53-null mice which develop early onset spontaneous tumors, all of the $p53^{3KR/3KR}$ mice remain tumor free [97]. These results indicate that the apoptosis, growth arrest, and senescence functions of p53 are dispensable for

p53 to execute its tumor suppressive effect and other aspects of p53 function are sufficient to suppress tumor formation. Notably, $p53^{3KR}$ retains the ability to regulate energy metabolism and reactive oxygen species production through transcription regulation of metabolic targets such as TIGAR, GLS2, and GLUT3 [97]. Our most recent study also demonstrated that $p53^{3KR}$ retains the capacity to promote ferroptosis, a non-apoptotic, iron-dependent and oxidative cell death, through repressing the expression of *SLC7A11*, a key component of the cystine/glutamate antiporter [81]. Moreover, our study in Section 3 clearly indicated that *SAT1*, a novel p53-regulated gene in polyamine metabolism and ferroptosis, can also be induced by $p53^{3KR}$. These results imply that ferroptotic cell death pathway may constitute a potential mechanism of p53-mediated tumor suppression.

Notably, p53 also regulates mTOR signaling pathway. mTOR is a central regulator of cell growth and proliferation in response to nutrients, growth factors, energy metabolism and hypoxia [158]. It is an evolutionarily conserved serine-threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family [159]. mTOR is regulated by a group of tumor suppressors and oncogenes, and alterations in mTOR signaling pathway are associated with cancer development. In response to growth factors such as IGF-1, mTOR is activated through a kinase cascade consisting of PI3K, PDK1, and AKT, which is negatively regulated by tumor suppressor PTEN [91]. Whereas in response to low energy levels, mTOR is inhibited by

adenosine monophosphate (AMP)-dependent kinase (AMPK), which is activated by tumor suppressor LKB1 [160]. Additionally, hypoxia signaling also inhibits mTOR through activating the expression of DNA damage inducible transcript 4 (DDIT4) [161]. Notably, the regulations of mTOR through these signaling pathways are all via tumor suppressor tuberous sclerosis 1 and 2 complex (TSC1/2), which negatively regulates mTOR. Mutations in TSC2 leads to tuberous sclerosis syndrome associated with an increased risk of renal cell carcinoma (RCC) [160]. PTEN mutation or deletion, which result in constitutive activation of AKT and mTOR signaling, are found in a number of cancers including hepatocellular carcinoma, glioblastoma, lung carcinoma and melanoma [162, 163]. It is well-known that mTOR activation increases the phosphorylation of its downstream targets, and regulates a variety of cellular processes including translation, ribosome biogenesis, and inhibition of autophagy, all of which promote cell growth and proliferation. The p70S6 kinase (p70S6K) and eIF4E binding protein 1 (4EBP1) are two most well-characterized mTOR targets that are involved in translation regulation. mTOR phosphorylates p70S6K and 4EBP1, and increases the level of translation [164]. Interestingly, genes coding for S6K and eIF4E are found be to be amplified in several types of human cancers, and overexpression of eIF4E results in increased frequency of tumor formation, suggesting that mTOR signaling pathway is closely related with cancer development [165-167]. Notably, the expression of several negative regulators of mTOR, including DDIT4, TSC2, PTEN, AMPKβ1,

and IGF-BP3, are directly activated by p53, resulting in inhibition of mTOR by p53 [92]. Moreover, recent studies have revealed that *SESN2* (a gene coding for Sestrin2) is a p53-inducible target upon genotoxic stress that inhibits mTOR signaling through activation of AMPK and TSC2 phosphorylation [168].

Given that cell cycle arrest and pro-apoptotic target genes of p53 are regulated by acetylation at K120 and K164, we postulated that additional modifications may exist that are crucial for the regulation of non-canonical p53 targets. In this section, we identified two novel p53 acetylation sites at lysine K101 and K139 within the human p53 DNA-binding domain. Both lysine residues are evolutionarily conserved and are homologous to lysine K98 and K136 in mouse p53. Ablation of K98 or K136 acetylation alone has very modest effects on p53-mediated transcription activation. However, simultaneous loss of acetylation at K98 or/and K136 with K117/161/162 ($p53^{4KR98}$, $p53^{4KR136}$, $p53^{5KR}$) significantly abrogated p53-mediated activation of *TIGAR* and *SAT1*, and repression of *SLC7A11*. As a result, $p53^{4KR98}$, $p53^{4KR136}$, and $p53^{5KR}$ are all defective in inducing ferroptosis upon oxidative stress. Similar to $p53^{-/-}$ mice, all of the $p53^{5KR/5KR}$ mice developed spontaneous tumors within 11 months of age. Interestingly, although most of the $p53^{4KR98/4KR98}$ and $p53^{4KR136/4KR136}$ mice developed spontaneous tumors within 18 months of age, their tumor formation was significantly delayed for 6 months comparing with $p53^{5KR/5KR}$ mice. Notably, although $p53^{4KR98}$ has lost the ability to induce ferroptosis, it still retains the activity to

inhibit mTOR signaling through activating the expression of two mTOR negative regulators, Sestrin2 and DDIT4. Our studies suggest that p53-mediated ferroptosis is a critical barrier to prevent tumor formation. However, in the absence of cell cycle arrest, apoptosis and ferroptosis, p53-mediated regulation of the nutrient-sensing mTOR signaling pathway may play an important role in delaying tumor development.

4.2 Materials and Methods

Protein purification and mass spectrometry

Acetylated p53 protein was purified for mass spectrometry analysis to identify novel acetylation sites. In general, H1299 cells were co-transfected with vectors expression Flag-tagged p53, HA-CBP and Tip60 for 16 hr, and then treated with 5 mM nicotinamide and 1 μM TSA for 8 hr. Cells were harvested and lysed in Flag-lysis buffer with fresh proteinase inhibitor cocktail, 10 mM nicotinamide, and 2 mM TSA. The cell extracts were then immunoprecipitated with M2 beads and eluted with Flag peptide (Sigma). The eluted sample was resolved on 4%–20% Tris-glycine SDS-PAGE (Invitrogen), and the p53 bands were excised and subjected to mass spectrometric analysis.

Plasmids, transfection and immunoprecipitation

The plasmids expressing $p53^{K101R}$, $p53^{8KR}$ (K120/164R+6KR), $p53^{9KR}$ (K120/139/164R+6KR), and mouse $p53^{K98R}$ were derived from pCIN4-Flag-p53 or Topo-Flag-mp53 by mutagenesis using QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's protocol. Tet-on inducible mouse p53 mutant constructs were generated by sub-cloning mutant p53 from Topo vector into pTRIPZ tetracycline-inducible vector (Thermo). All transfections were performed using Lipofectamine2000 (Invitrogen) according to manufacturer's protocol. To detect p53 acetylation on lysine K101 or K139, p53

protein was immunoprecipitated with M2 beads, eluted with Flag peptide, resolved on SDS-PAGE, and detected by α-AcK101 p53 antibody or α-AcK139 p53 antibody.

Cell culture and stable lines

All cells were cultured in 37°C incubator with 5% CO_2. H1299 cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units per ml penicillin and 100 μg/ml streptomycin (all Gibco). MEFs were generated from day 13.5 embryos according to standard procedures. FBS used for MEFs was heat-inactivated and supplemented with 1% non-essential amino acids. Tet-on inducible H1299 stable cell lines expressing mouse p53 mutants were generated by transfecting pTRIPZ-mp53 mutant constructs into H1299 cells, followed by puromycin selection (1μg/mL) for 14 days. Established Tet-on inducible cell lines were maintained in DMEM with 10% Tet-free FBS (Clonetech). To induce the expression of p53, 0.5 μg/mL of tetracycline was added into culture medium.

Western blotting and antibodies

Cell lysates were prepared in Flag lysis buffer with fresh protease inhibitor cocktail. Protein extracts were analyzed by western blotting according to standard protocols using primary antibodies specific for human p53 (DO-1, Santa Cruz), α-AcK101 p53 (custom made by PTM), α-AcK139 p53 (custom made by PTM), mouse p53 (CM5, Leica biosystems), MDM2 (Ab5, Millipore), TIGAR (E2, Santa Cruz), p21 (SX118, Santa Cruz), Actin (A3853, Sigma-Aldrich),

Phospho-p70 S6 Kinase (Thr389, Cell Signaling Technologies), p70 S6 Kinase (49D7, Cell Signaling Technologies), Phospho-4E-BP1 (Ser65, Cell Signaling Technologies), 4E-BP1 (Cell Signaling Technologies), and DDIT4 (10638-1-AP, Proteintech). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (GE Healthcare) were used and signals were detected on autoradiographic films with Pierce ECL western blotting detection system or SuperSignal West Dura reagents (Thermo scientific).

RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol Reagent (Life Technologies) according to manufacturer's protocol. cDNA was synthesized from total RNA using M-MulV Reverse Transcriptase kit (NEB). PCR analysis was performed using Applied Biosystems 7500 Fast System. For the qRT-PCR analysis of human transcripts the following primers were used:

TIGAR forward 5' -CTCAAGACTTCGGGAAAGGA- 3',

TIGAR reverse 5' -GGTGTAAACACAGGGCACTCTT- 3',

SAT1 forward 5' -CCGTGGATTGGCAAGTTATT- 3',

SAT1 reverse 5' -TCCAACCCTCTTCACTGGAC-3',

SLC7A11 forward 5' -ATGCAGTGGCAGTGACCTTT- 3',

SLC7A11 reverse 5' -GGCAACAAAGATCGGAACTG- 3',

SESN2 forward 5' -CGCTTTGAGCTGGAGAAGTC- 3',

SESN2 reverse 5’ -TCCACAAAGCACAGCATGTC- 3’,

DDIT4 forward 5’ -CTGGACAGCAGCAACAGTG- 3’,

DDIT4 reverse 5’ -ACACCCCATCCAGGTAAGC- 3’,

GAPDH forward 5’ -ATCAATGGAAATCCCATCACCA- 3’,

GAPDH reverse 5’ -GACTCCACGACGTACTCAGCG- 3’.

For the qRT-PCR analysis of mouse transcripts the following primers were used:

Sesn2 forward 5’ -CAGCGCTTTCATTCCAGTG- 3’,

Sesn2 reverse 5’ -CCGGGTGTAGACCCATCA- 3’,

Ddit4 forward 5’ -CCAGAGAAGAGGGCCTTGA- 3’,

Ddit4 reverse 5’ -CCATCCAGGTATGAGGAGTCTT- 3’.

Cell death count, drug and inhibitors

Cell death was determined by trypan blue assay. Erastin was used at a concentration of 1μM in H1299 cells, DNA damaging reagent Etoposide was used at a concentration of 20μM in MEFs.

4.3 Results and Discussion

4.3.1 p53 is acetylated at lysine K101 and K139 residues by CBP and Tip60

To screen for novel p53 acetylation sites, we purified ectopically expressed p53 protein in p53-null H1299 cells with the presence of two major p53 acetyltransferases CBP and Tip60, and analyzed the post-translational modifications of p53 using mass spectrometry. Our mass spectrometry data revealed two previously uncharacterized p53 acetylation sites at lysine residue K101 and K139, both located in p53 DNA-binding domain (Figure 4.1A and 4.2A). K101 and K139 lysine residues are both evolutionarily conserved, and are homologous to the K98 and K136 lysine residues in mouse p53 respectively (Figure 4.1B and 4.2B). To validate the acetylation at K101, we generated a rabbit monoclonal antibody specifically against p53 acetylated at lysine K101. We first transfected H1299 p53-null cells with vectors expressing either Flag-tagged wild-type p53 or K101R p53 in the absence or presence of acetyltransferases CBP or Tip60, and the tagged p53 was immunoprecipitated and resolved on SDS-PAGE for western blot analysis. The site-specific AcK101-p53 antibody only recognized wild-type p53 acetylated by CBP, but not acetylation-deficient K101R p53 mutant (Figure 4.1C). Furthermore, Tip60 does not acetylate p53 at K101 either (Figure 4.1C). A similar experiment was performed using mouse p53, and the AcK101-p53 antibody also recognized acetylation of mouse p53 at K98 in the presence of CBP (Figure 4.1D). Together, these results indicate that K101 lysine

residue of p53 is a bona fide acetylation site of CBP. Similarly, to validate the acetylation at K139, we generated a rabbit polyclonal antibody against acetylated p53 at lysine 139 (AcK139-p53). Because the AcK139-p53 antibody has cross-reactivity with acetylated lysine K120, K164, and six C-terminal acetylation sites, we then compared the signal detected between the acetylation-deficient mutant $p53^{8KR}$ (K120/164R+6KR) and $p53^{9KR}$ (K120/139/164R+6KR). p53-null H1299 cells were transfected with either Flag-tagged $p53^{8KR}$ or $p53^{9KR}$ in the absence or presence of acetyltransferase Tip60, and the tagged p53 were immunoprecipitated for western blot analysis on SDS-PAGE. As shown in Figure 4.2C, the AcK139-p53 antibody only recognized $p53^{8KR}$ acetylated by Tip60, but not $p53^{9KR}$. This result indicates that K139 of p53 is acetylated by Tip60.

Figure 4.1

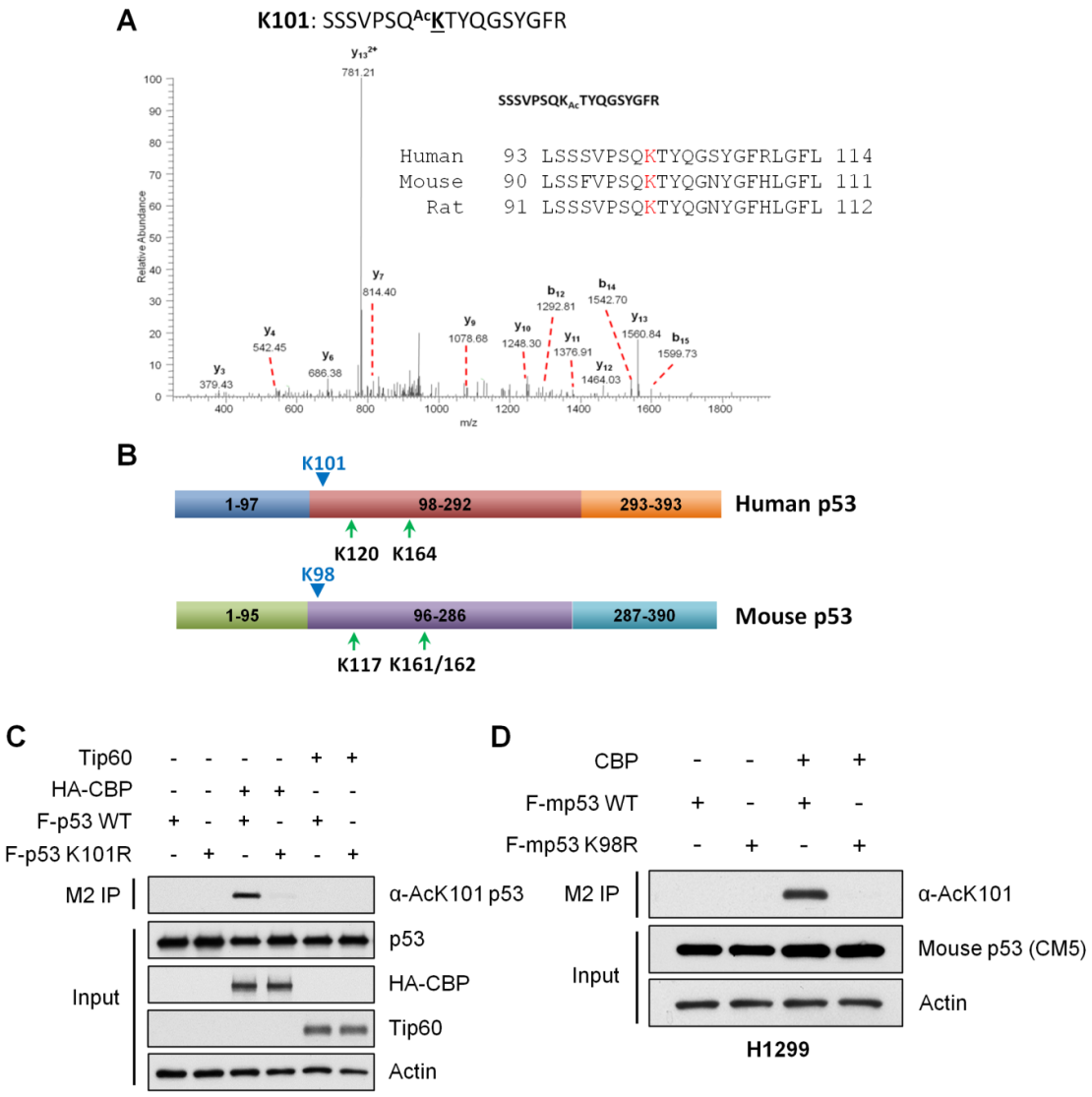

Figure 4.1. Acetylation of Human p53 at K101 and Mouse K98 by CBP. (A) Mass spectrometry of purified p53 protein in the presence of CBP uncovered acetylation of p53 at the K101 lysine residue. **(B)** Cartoon figure depicting the K101 lysine residue (K98 for mouse p53) located in the N-terminal end of the DNA-binding domain. **(C)** H1299 cells were transfected with vectors expressing either Flag-tagged human wild-type or K101R mutant p53 in the presence of CBP and Tip60. Whole-cell lysates were collected, and Flag-tagged p53 was immunoprecipitated using M2 Flag beads. K101-acetylated p53 was detected using site-specific antibody. **(D)** Similar experiment to (C) was performed using Flag-tagged mouse wild-type or K98R mutant p53 in the presence of CBP, and p53 acetylation at K98 was detected using the same site-specific antibody against acetylated K101.

Figure 4.2

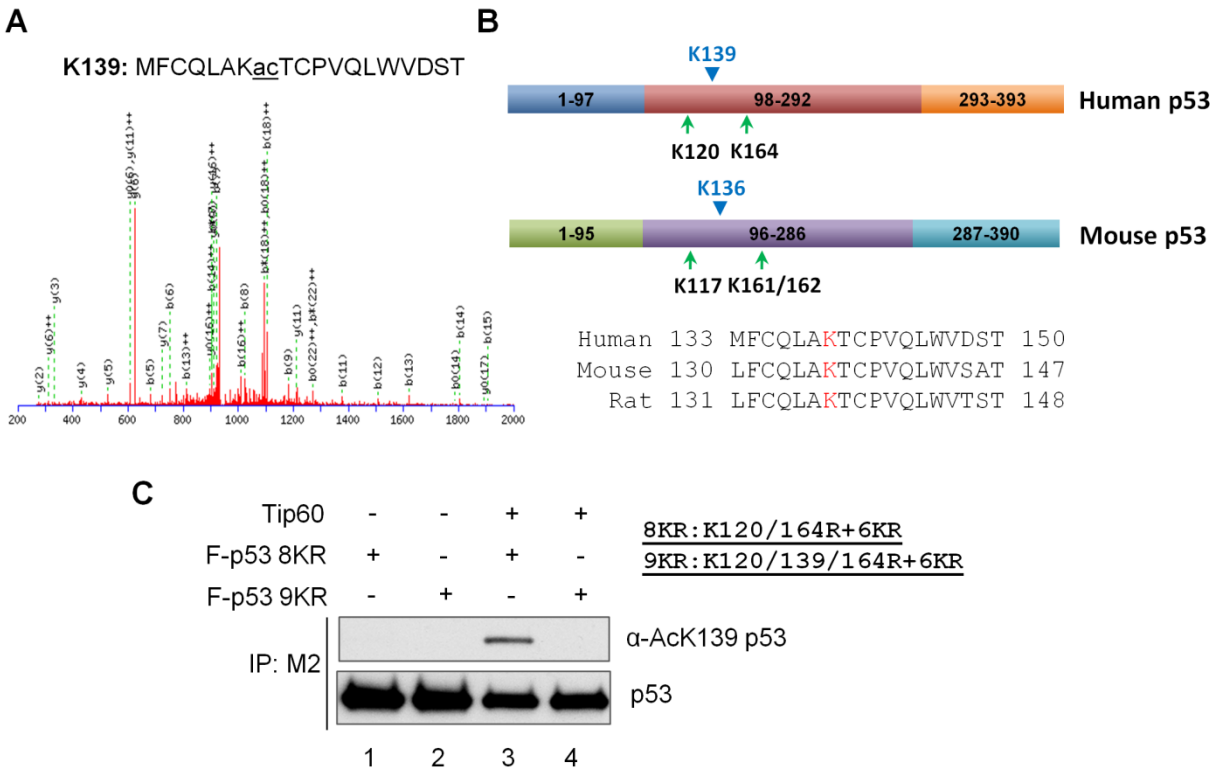

Figure 4.2. Lysine K139 in Human p53 (K136 in Mouse p53) is a novel acetylation site acetylated by Tip60. (A) Mass spectrometry of purified p53 protein in the presence of CBP and Tip60 uncovered acetylation of p53 at the K139 lysine residue. **(B)** Schematic representations of human and mouse p53 protein depicting the K139 lysine residue (K136 for mouse p53) located in DNA-binding domain. **(C)** H1299 cells were transfected with vectors expressing either Flag-tagged human 8KR or 9KR mutant p53 in the presence of Tip60. Flag-tagged p53 was immunoprecipitated using M2 beads, and K139-acetylated p53 was detected with site-specific antibody AcK139-p53.

4.3.2 Simultaneous loss of acetylation at K117/161/162 with K98 or K136 impairs p53 transcriptional activities

Our previous studies have shown that ablation of acetylation in human K120/164 or mouse K117/161/162 abrogates p53's ability to transactivate target genes involved in growth arrest, apoptosis, and senescence. However, the acetylation-deficient mutant mouse $p53^{3KR}$ (K117/161/162R) retains the regulation on metabolic targets such as *TIGAR*, and ferroptosis target S*LC7A11*. Thus, we wish to investigate whether ablation of K98 or K136 acetylation would further impair p53's transcription activity. Most recently, we have revealed that loss of K98 acetylation ($p53^{K98R}$) alone has very modest effect on p53-mediated transactivation. However, simultaneous mutations at all four acetylation sites K98/117/161/162 ($p53^{4KR98}$) completely abolish its ability to regulate *TIGAR* and *SLC7A11*. Similarly, to evaluate the effect of K136 acetylation, H1299 cells were transfected with vectors expressing wild-type p53, $p53^{3KR}$, $p53^{K136R}$, or $p53^{4KR136}$ (3KR+K136R), and cell lysates were collected and resolved on SDS-PAGE for western blot analysis. As a result, ectopic expression of $p53^{K136R}$ showed comparable transcriptional activity with wild-type p53 on target genes such as *MDM2*, *TIGAR*, and *p21* (Figure 4.3A). However, simultaneous mutations of K117/161/162 and K136 result in significant defect in transactivating Mdm2 and Tigar expression (Figure 4.3A). Furthermore, we have established Tet-on inducible H1299 stable cell lines conditionally expressing various p53

acetylation-deficient mutants $p53^{3KR}$, $p53^{4KR98}$, $p53^{4KR136}$, and $p53^{5KR}$ (3KR+K98R+K136R). The p53 mutants were then induced by the addition of tetracycline (Tet-on), and downstream targets transcription was examined using qRT-PCR. As shown in Figure 4.3B-D, $p53^{4KR98}$, $p53^{4KR136}$, and $p53^{5KR}$ all lost the ability to transcriptionally activate the expression of *TIGAR*, *SAT1*, and repress the expression of *SLC7A11*. Interestingly, our previous data demonstrated that loss of K98 acetylation does not affect DNA binding, since wild-type mouse p53, $p53^{3KR}$, and $p53^{4KR98}$ were all able to recruit to the *TIGAR* gene promoter to the similar extent. To further assess the DNA-binding properties of $p53^{4KR136}$ and $p53^{5KR}$, we performed chromatin immunoprecipitation assay in H1299 cells transfected with various acetylation-deficient mutants. As a result, both $p53^{4KR136}$ and $p53^{5KR}$ were able to bind to *TIGAR* and *SLC7A11* gene promoters to the same extent as $p53^{3KR}$ (Figure 4.4). Taken together, these data indicate that K98 and K136 acetylation in the p53 DNA-binding domain control p53 transcriptional activity without affecting DNA binding capacity.

Figure 4.3

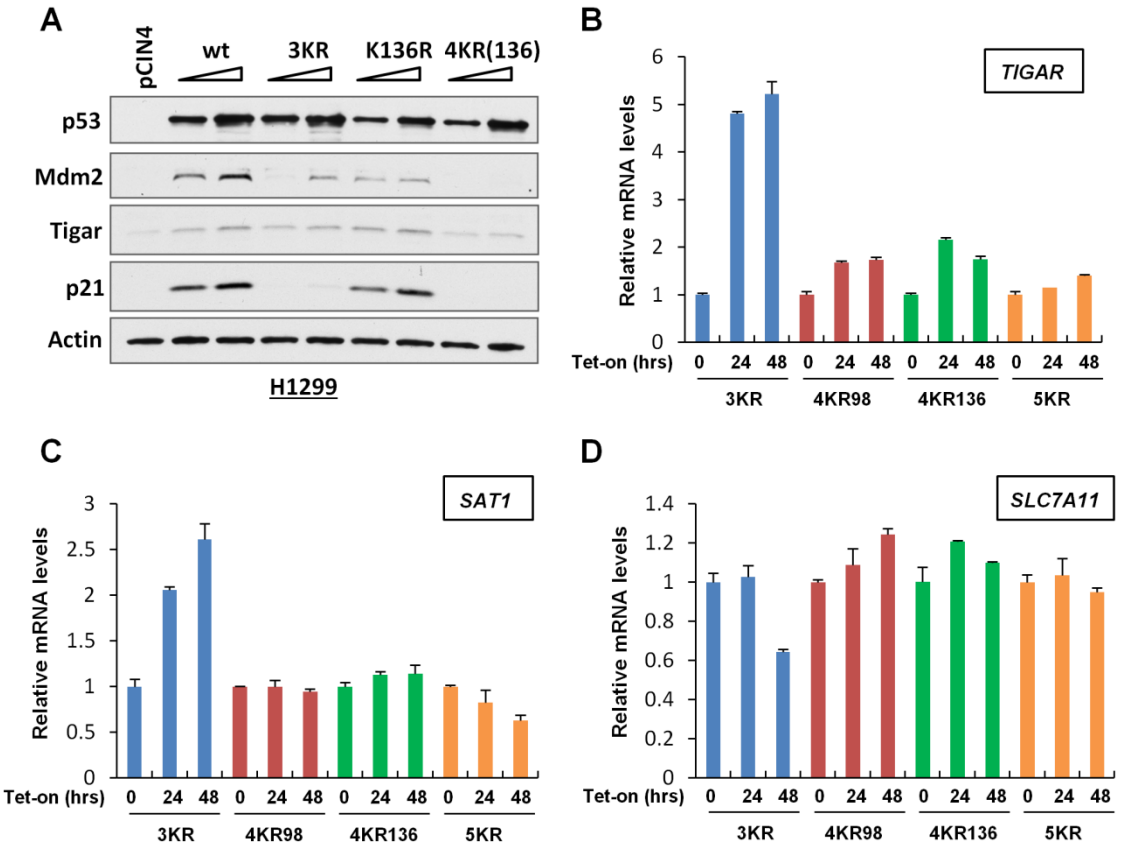

Figure 4.3. Simultaneous loss of acetylation at K117, K161, K162 with K98 or/and K136 abolishes p53's ability to regulate *TIGAR*, *SAT1*, and *SLC7A11*. (A) H1299 cells were transfected with empty vector and vectors expressing wild-type, 3KR, K136R, or 4KR136 mutant p53. Expression of MDM2, TIGAR, and p21 were detected via western blot analysis. **(B-D)** H1299 inducible p53 stable cell lines expressing wild-type, 3KR, 4KR98, 4KR136, or 5KR p53 were treated with doxycycline for indicated times, and transcription levels of p53 target genes *TIGAR*, *SAT1*, and *SLC7A11* were determined by qRT-PCR.

Figure 4.4

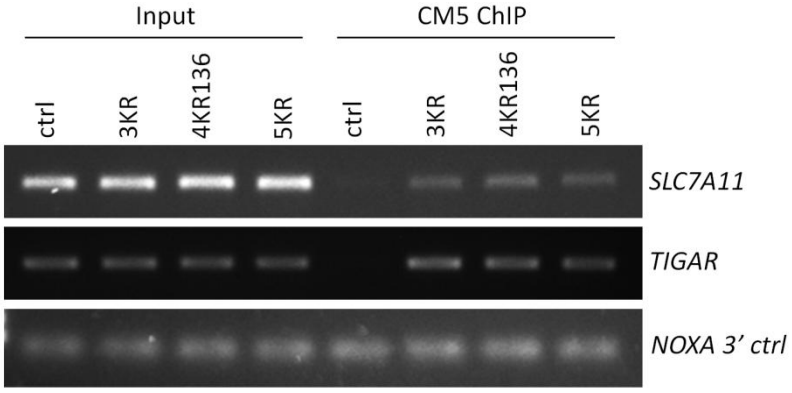

Figure 4.4. Effect of K98 and K136 acetylation on DNA binding capacity of p53. (A) ChIP assay was performed in H1299 cells transfected with empty vector, or vectors expressing $p53^{3KR}$, $p53^{4KR136}$, or $p53^{5KR}$. The immunoprecipitated DNA was amplified by PCR using primers that span the p53-binding region on *TIGAR* and *SLC7A11* promoter, and the PCR products were resolved on agarose gel.

4.3.3 p53-mediate ferroptotic response is abrogated in $p53^{4KR136}$ and $p53^{5KR}$

Our recent study has revealed that $p53^{4KR98}$ is defective in p53-mediated ferroptotic response. To elucidate whether $p53^{4KR136}$ and $p53^{5KR}$ also lost the ability to induce ferroptosis, we performed ferroptosis assay in Tet-on inducible cell lines treated with ferroptosis inducer Erastin. As expected, H1299 cells expressing $p53^{3KR}$ underwent significant ferroptosis upon Erastin treatment. However, cells expressing $p53^{4KR136}$ and $p53^{5KR}$ were resistant to Erastin and did not result in any cell death (Figure 4.5). This result indicates that p53-mediate ferroptotic response is also abrogated in $p53^{4KR136}$ and $p53^{5KR}$.

Figure 4.5

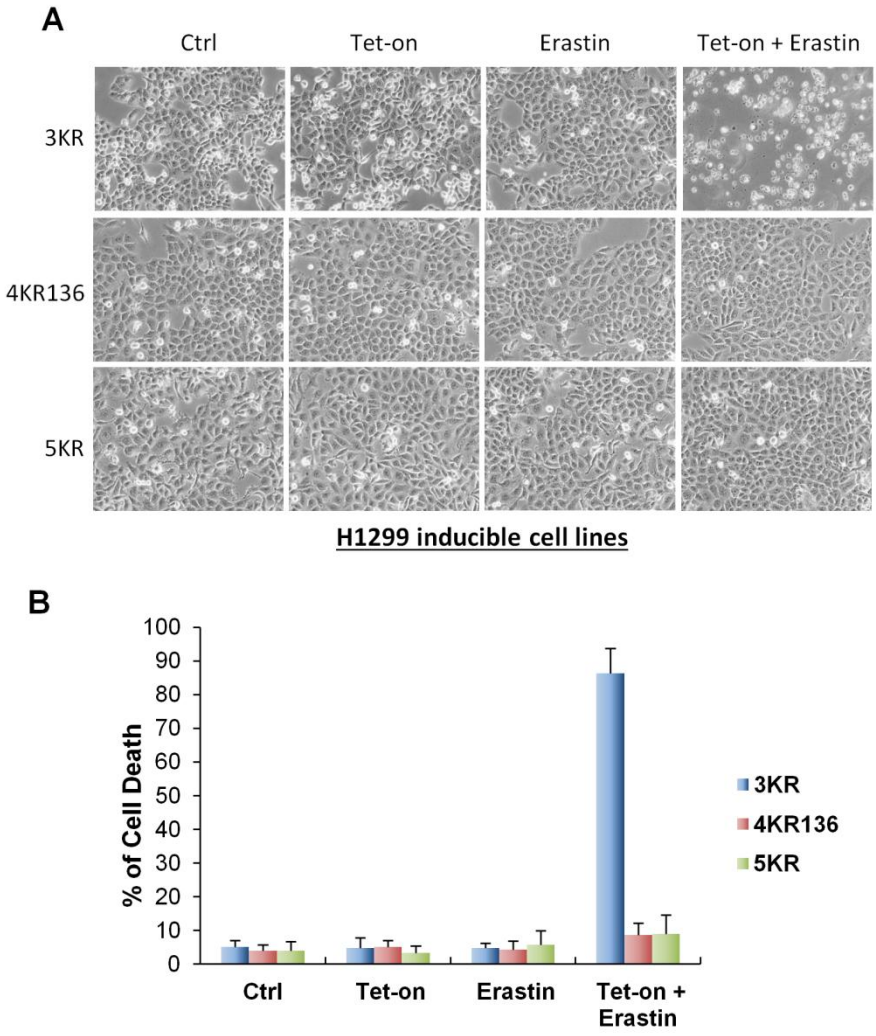

Figure 4.5. $p53^{4KR136}$ and $p53^{5KR}$ lost the ability to regulate ferroptosis upon oxidative stress. **(A)** Representative phase-contrast images of Tet-on inducible H1299 cells expressing 3KR, 4KR136 or 5KR p53 treated with doxycycline (Tet-on), Erastin, or doxycycline + Erastin (Tet-on + Erastin) for 48 hr. **(B)** Quantification of ferroptotic cell death in (A) from three technical triplicates (mean ± s.d.) is shown.

4.3.4 *p53*$^{4KR98/4KR98}$ and *p53*$^{4KR136/4KR136}$ knock-in mice lost intact tumor suppression, but displayed delayed tumor onset comparing to *p53*$^{5KR/5KR}$ mice

To ascertain whether ferroptosis or other non-canonical functions of p53 are required for tumor suppression, we generated *p53*$^{4KR98/4KR98}$, *p53*$^{4KR136/4KR136}$, and *p53*$^{5KR/5KR}$ knock-in mice, and compared their spontaneous tumor formation with *p53*$^{+/+}$, *p53*$^{3KR/3KR}$, and *p53*$^{-/-}$ mice. Notably, although 3 of the 27 *p53*$^{3KR/3KR}$ mice developed tumors, sequence analysis revealed that additional de novo mutations were acquired in the tumor tissues of these three *p53*$^{3KR/3KR}$ mice, indicating that p53^{3KR} retains intact tumor suppression activity [97]. Surprisingly, unlike *p53*$^{3KR/3KR}$ mice, 72% of the *p53*$^{4KR98/4KR98}$ mice, 55% of the *p53*$^{4KR136/4KR136}$ mice, and all of the *p53*$^{5KR/5KR}$ mice developed spontaneous tumors and died before 18 months of age (Figure 4.6). This result indicates that p53-mediated ferroptosis may function as a critical barrier to prevent tumor formation in the absence of cell cycle arrest, apoptosis, and senescence in *p53*$^{3KR/3KR}$ mice. However, upon further loss of p53-mediate ferroptotic responses, *p53*$^{4KR98/4KR98}$, *p53*$^{4KR136/4KR136}$, and *p53*$^{5KR/5KR}$ mice result in loss of intact tumor suppression.

Notably, the average time of the tumor onset in *p53*$^{4KR98/4KR98}$ and *p53*$^{4KR136/4KR136}$ mice was significantly delayed for 6 months comparing with that in *p53*$^{5KR/5KR}$ mice (Figure 4.6). This result suggests that p53^{4KR98} and p53^{4KR136} mutants still have some remaining functions that may play a role in delaying tumorigenesis.

Figure 4.6

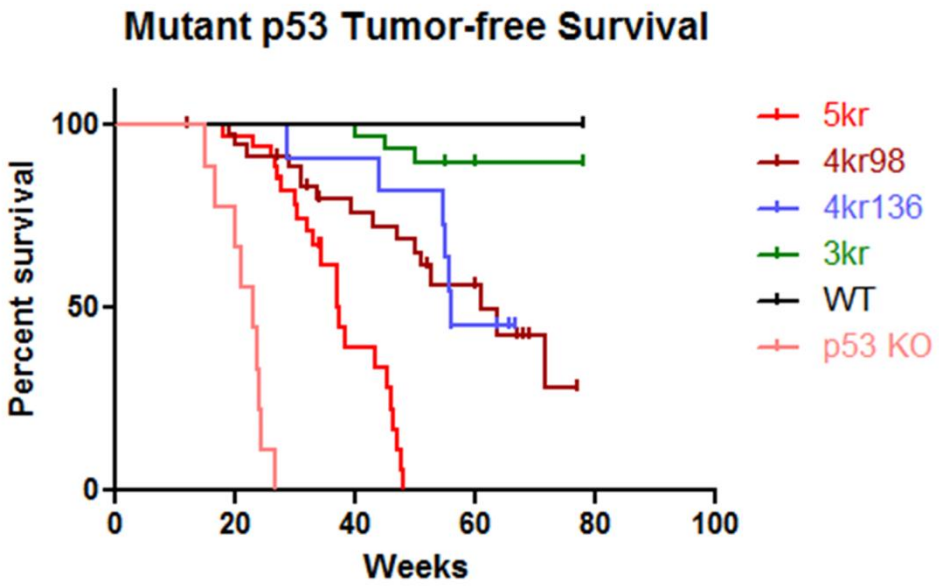

Figure 4.6. *p53*$^{4KR98/4KR98}$ and *p53*$^{4KR136/4KR136}$ knock-in mice lost intact tumor suppression, but displayed delayed tumor onset comparing to *p53*$^{5KR/5KR}$ mice. (Data from Dr. Nin Kong) Kaplan-Meier survival curves of *p53*$^{+/+}$ (n=16), *p53*$^{3KR/3KR}$ (n=27), *p53*$^{4KR98/4KR98}$ (n=46), *p53*$^{4KR136/4KR136}$ (n=11), *p53*$^{5KR/5KR}$ (n=35), and *p53*$^{-/-}$ (n=19) mice before 18 months of age.

4.3.5 Regulation of mTOR signaling pathway is retained by $p53^{4KR98}$, but not by $p53^{5KR}$

Notably, $p53^{4KR98}$ and $p53^{4KR136}$ have very similar transcriptional activities and tumor suppressive properties, indicating that acetylation at lysine K98 and K136 may have functional redundancies. To elucidate which remaining targets of p53 are retained in $p53^{4KR}$, we compared gene expression profiles between $p53^{4KR98/4KR98}$ and $p53^{5KR/5KR}$ MEFs upon the treatment of DNA damaging drug Etoposide using RNA-seq. RNA-seq result uncovered two target genes- *SESN2* and *DDIT4*, that can still be transactivated by $p53^{4KR98}$, but not by $p53^{5KR}$. We then further validated the transcriptional regulation of *SESN2* and *DDIT4* by various p53 acetylation-deficient mutants in H1299 Tet-on inducible cell lines and MEFs. mRNA expression of *SESN2* and *DDIT4* upon $p53^{3KR}$ and $p53^{4KR98}$ induction were both elevated in a time-dependent manner, whereas the expression of these two genes after $p53^{5KR}$ induction remained unchanged (Figure 4.7A and 4.7B). Similarly, $p53^{4KR98/4KR98}$ MEFs treated with Etoposide for 24 hours showed an elevation of both *Sesn2* and *Ddit4* transcript levels; whereas $p53^{5KR/5KR}$ MEFs undergoing the same treatment had no change in *Sesn2* and *Ddit4* mRNA levels (Figure 4.7C and 4.7D). Since Sestrin2 and DDIT4 are both negative regulators of mTOR, we speculate if $p53^{4KR98}$ also repress mTOR signaling. Phosphorylation of p70S6K and 4EBP1 are induced by mTOR, and are used as two important indicators of mTOR activation. As shown in Figure 4.8, Etoposide treatment for 24 hours significantly reduced the levels of p70S6K

phosphorylation and 4EBP1 phosphorylation in *p53*$^{4KR98/4KR98}$ MEFs, but not in *p53*$^{5KR/5KR}$ MEFs. Taken together, these results demonstrate that p53^{4KR98} retains the ability to inhibit mTOR signaling, and this remaining activity may contribute to the observed delay of tumorigenesis in *p53*$^{4KR98/4KR98}$ knock-in mice.

Figure 4.7

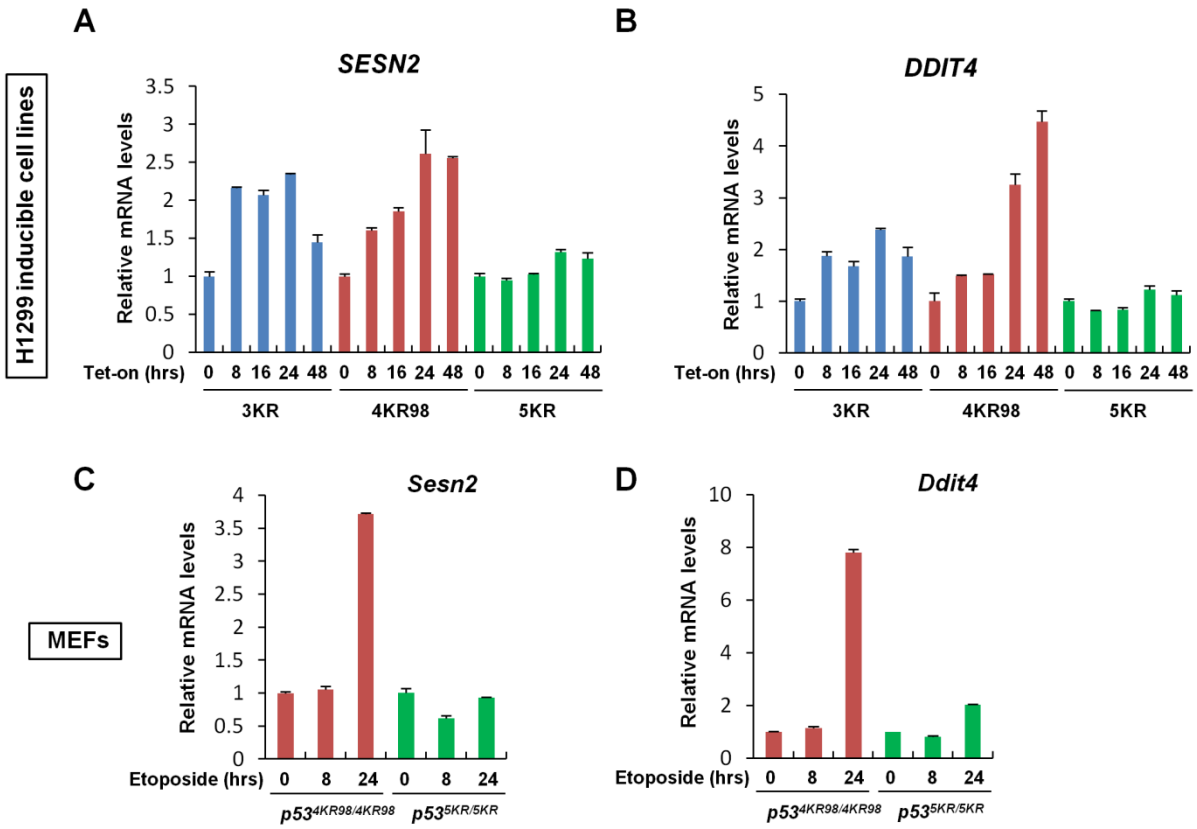

Figure 4.7. Regulation of *SESN2* and *DDIT4* gene expression are retained by p53^{4KR98}, but not by p53^{5KR}. (A and B) H1299 inducible p53 stable cell lines expressing 3KR, 4KR98, or 5KR p53 were treated with doxycycline for indicated times, and expression levels of *SESN2* and *DDIT4* were determined by qRT-PCR. **(C and D)** qRT-PCR analysis of *Sesn2* and *Ddit4* mRNAs in *p53*$^{4KR98/4KR98}$ and *p53*$^{5KR/5KR}$ MEFs either untreated or treated with 20μM Etoposide (Etp) for 8 hr and 24 hr. All mRNA expression levels were normalized with *GAPDH*.

Figure 4.8

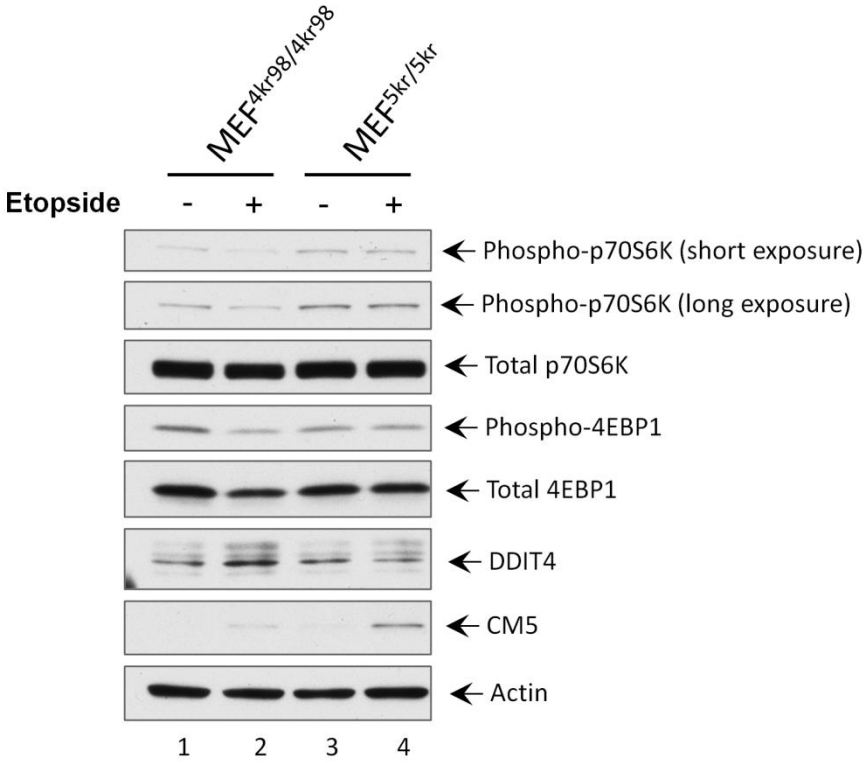

Figure 4.8. Regulation of mTOR signaling pathway is retained by p53^{4KR98}, but not by p53^{5KR}. *p53$^{4KR98/4KR98}$* and *p53$^{5KR/5KR}$* MEFs were treated with 20μM Etoposide for 24 hr, and crude cell lysates were obtained for the western blot analysis using antibodies against phospho-p70S6K, p70S6K, phospho-4EBP1, 4EBP1, DDIT4, CM5 (mouse p53) and Actin.

CHAPTER 5

Discussion and Future Directions

p53 is a bona fide tumor suppressor and serves as a guardian of the genome to prevent malignant transformation. It mainly functions as a transcription factor to monitor signaling pathways through transcriptional regulation of target genes involved in cell cycle arrest, apoptosis, and senescence [121]. In our studies, we have uncovered diverse array of p53-mediated cellular functions, including serine metabolism, polyamine metabolism, ferroptosis, and regulation of mTOR signaling pathway, which may contribute significantly to the tumor suppressive activity of p53.

5.1 PHGDH is a novel p53 target in serine metabolism

Although p53 is well known for its transcriptional activation function, there are more and more studies suggesting that p53 is also able to repress gene transcription, and that such regulation is important for its ability to regulate cell death and metabolism [169, 170]. In this study, we identified that *PHGDH*, the key serine metabolism gene, is a repression target of p53 and mediates its apoptotic response upon serine starvation. PHGDH has recently been shown to play an important role in tumor development, and is frequently amplified or over-expressed in melanoma, a type of skin cancer that rarely has p53 mutations. Here in melanoma cell lines, we demonstrated that PHGDH mRNA and protein levels were significantly down-regulated in response to Nutlin-3 and DNA-damaging agents in a p53-dependent manner. Furthermore, we found a p53 consensus sequence in the *PHGDH* promoter region, and therefore, p53 directly

binds to this response element to repress gene transcription. Although the precise mechanisms of transcription repression remain largely unknown, a number of studies suggest that p53 represses gene transcription through interfering with the functions of DNA-binding transcriptional activators, or recruiting transcriptional repressors, such as histone deacetylases [171]. Therefore, it would be interesting to explore whether histone deacetylase inhibitors, such as trichostatin A (TSA), can block p53-mediated transcriptional repression of *PHGDH*.

PHGDH catalyzes the first and rate-limiting step in the serine synthesis pathway [116]. In light of the recent findings that some melanoma and breast cancer cell lines with amplified PHGDH locus are highly dependent on PHGDH expression for their proliferation [110, 111], PHGDH inhibitors are being developed (in preclinical studies) to treat these types of cancers [172]. In our study, we revealed that a combination of PHGDH depletion and serine starvation not only inhibits proliferation, but also induces significant apoptosis in melanoma cells. We also demonstrated that the mechanisms underlying apoptosis triggered by inhibiting PHGDH in the presence of serine starvation is mediated through ATF4-dependent, but p53-independent Puma and Noxa activation. These results imply that inhibitors of serine metabolism, when utilized in the setting of appropriate nutrient (specifically serine) restriction, may have therapeutic potential against cancer.

Given our results showing that PHGDH is a novel repression target of p53, activators of p53

can be used to inhibit PHGDH expression. Nutlin-3 is a non-genotoxic drug that specifically activates p53 transcription through disrupting its interaction with Mdm2. However, Nutlin-3 is not an ideal drug for eradicating melanoma cells that retain wild-type p53, because it only induces p53-mediated cell cycle arrest without triggering apoptosis [125]. Thus, the possibility of reactivating the apoptotic function of p53 in melanoma cell lines could have profound implications in future therapeutic directions [108, 109]. Here, we have identified a novel p53 downstream pathway that modulates serine metabolism, which can sensitize melanoma cells to apoptosis. As shown in the proposed model in Figure 5.1, activation of p53 in melanoma cell lines by Nutlin-3 can suppress PHGDH expression through transcriptional repression. Under normal condition in which serine is present, activation of p53 by Nutlin-3 only induces cell cycle arrest through p21 activation and PHGDH down-regulation. However, in metabolic stress condition in which serine is absent, suppression of PHGDH by Nutlin-3 can further promote apoptosis through ATF4-dependent Puma and Noxa activation. Meanwhile, our findings implied that small molecules that interrupt serine uptake may be combined with Nutlin-3 to enhance killing of human melanoma cells.

Although we have uncovered that p53 can negatively regulate oncogenic serine synthesis pathway through transcriptional repression of *PHGDH*, it is still unknown whether this regulation pathway contributes to p53-mediated tumor suppression. Notably, transcription

repression of *PHGDH* is partially retained by $p53^{3KR}$. Further investigations are required to determine if constitutive PHGDH overexpression in $p53^{3KR/3KR}$ mice (by crossing with *PHGDH* transgenic mice) could abrogate intact tumor suppression mediated by $p53^{3KR}$. Nevertheless, our study provided novel mechanistic insights into how p53 promotes apoptosis in cancer cells through the regulation of oncogenic serine metabolism pathway.

Figure 5.1

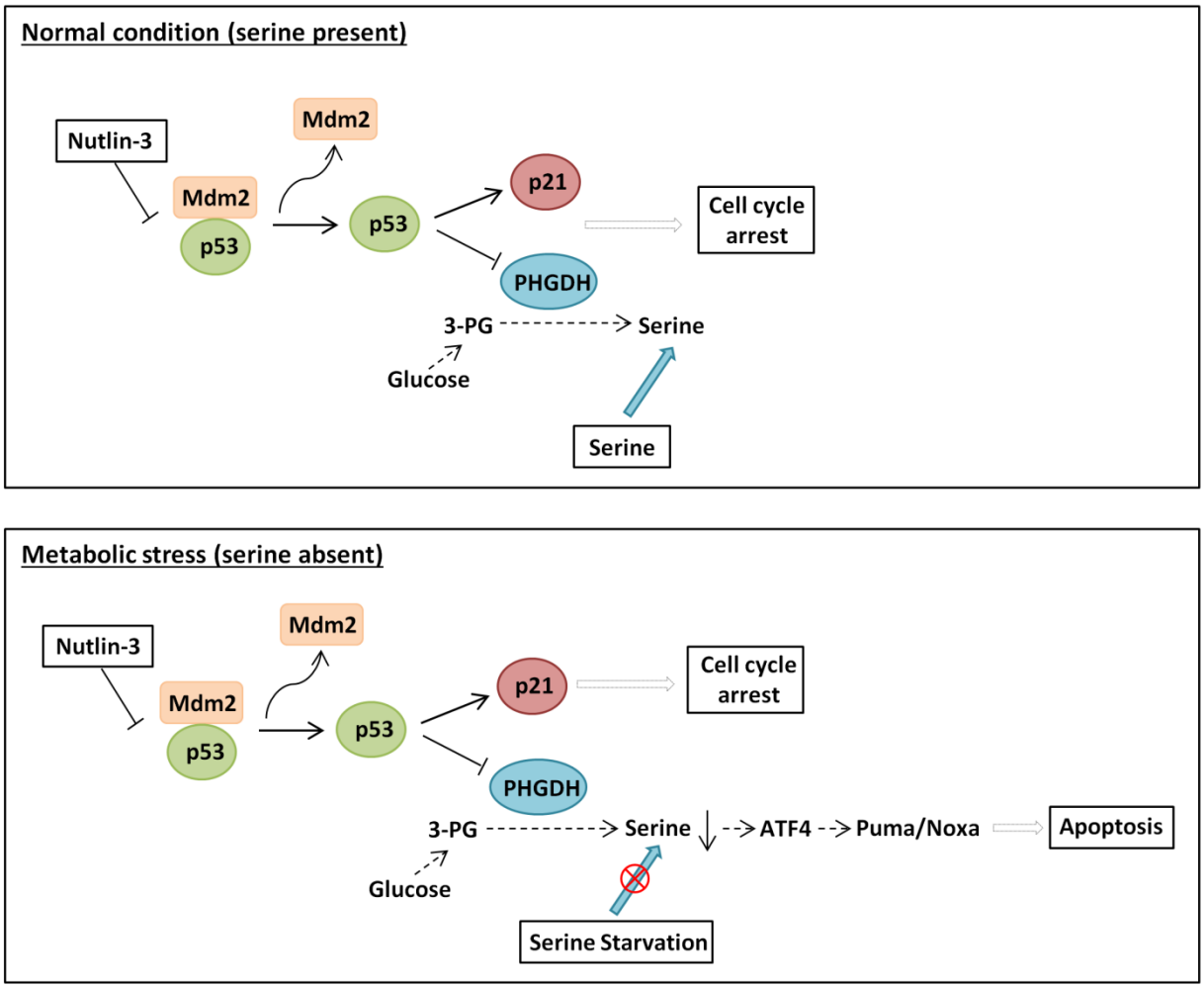

Figure 5.1. Proposed models demonstrating the differential effects of p53 on its apoptotic response through regulating PHGDH under normal condition or serine starvation.

5.2 Identification of SAT1 as a novel p53 target in polyamine metabolism and ferroptosis

Several recent studies have highlighted the importance of p53 in the regulation of cellular metabolism and oxidative stress response. In this study, we have provided evidence that linked p53 function to polyamine metabolism and ROS response through a novel metabolic target SAT1, an enzyme that catalyzes the rate-limiting step of polyamine catabolism. *SAT1* was initially identified as a p53 inducible gene from RNA sequencing of p53 wild-type melanoma cells treated with Nutlin, a small molecular that activates p53 through inhibiting p53's negative regulator MDM2. Subsequently, we revealed that *SAT1* is highly inducible by both Nutlin and DNA damage in various cancer cell lines, and the transcriptional regulation is dependent on p53. This is also consistent with the previous finding that *SAT1* is a highly inducible target by 5-fluorouracil (5-FU) [173]. ChIP-qPCR analysis revealed two p53 binding sites on the promoter region of *SAT1*, indicating that *SAT1* is a direct p53 target.

Further characterization of the functions of SAT1 was carried out in a tetracycline-inducible cell line expressing SAT1. Although the effects of SAT1 overexpression were previously described by other groups to cause a rapid cell growth arrest in Hela cells, as well as apoptosis in glioblastoma cells using transient transfection [138, 174], we observed neither of these phenomena in our inducible expression system. This may be attributable to the levels of SAT1 being expressed in cells, as a lower expression level of SAT1 induced by tetracycline may better

mimic the physiological condition compared to transient transfection. Meanwhile, our xenograft study clearly indicated that SAT1 possesses tumor suppressive properties. Although genetic alterations of *SAT1* gene have not been reported, both our analysis of *SAT1* expression levels in cancer patient samples and Oncomine database demonstrated that SAT1 is significantly down-regulated in a variety of human cancers. This is also consistent with the fact that cancer cells have elevated polyamine levels.

Although numerous metabolic targets of p53 have been identified, it is still not completely understood how metabolic functions contribute to p53-mediated tumor suppression. Intriguingly, our recent studies have revealed that ferroptosis, an iron-dependent and non-apoptotic cell death, provides another layer of protection against tumorigenesis [81]. In response to inappropriate levels of ROS, p53 sensitizes cells to ferroptosis by transcription repression of SLC7A11, a component of the cystine/glutamate antiporter [81]. Nonetheless, it remains largely unknown how ferroptosis is regulated, and what additional targets of p53 contribute to ROS-induced ferroptosis. In this study, we discovered that SAT1 significantly induced lipid peroxidation and ferroptosis upon ROS exposure. Moreover, our data indicates that p53-mediated transcriptional activation of *SAT1* is critical for ROS-induced ferroptosis, as knockout of *SAT1* significantly abrogated p53-induced ferroptosis upon ROS stress. Interestingly, although SAT1 failed to elicit growth arrest and apoptosis, elevation of ferroptosis maker *Ptgs2* was detected in xenograft

tumors harboring SAT1 induction. This further implies the importance of ferroptosis in suppression of tumor growth although future investigations are necessary to evaluate the precise role of ferroptosis on SAT1-mediated tumor suppression.

Ferroptosis is a mode of cell death that involves the production of both cytosolic and lipid ROS resulting from metabolic dysfunction [84, 150]. GPX4 is a glutathione peroxidase that catalyzes the reduction of lipid peroxides on cellular membrane. Lipid peroxidation and ferroptosis were observed in mouse xenografts harboring *Gpx4* knockout, highlighting the central regulating role of GPX4 in ferroptotic pathway [150]. However, we did not observe an expression level change of GPX4 in SAT1-induced ferroptotic cells, nor did the overexpression of SLC7A11 rescue cell death. Notably, we revealed that SAT1-induced ferroptosis is dependent on ALOX15, a lipoxygenase that catalyzes the peroxidation of arachidonic acid (AA). Our data demonstrated that SAT1 increases the expression of *ALOX15*, and ALOX15 inhibitor can completely rescue SAT1-induced ferroptosis. This is also consistent with the previous finding that ALOX15 is the core mediator that translates oxidative stress into lipid peroxidation and cell death [153]. Nonetheless, although we found a correlation between SAT1 and induced expression of *ALOX15*, the precise mechanism of how SAT1 regulates *ALOX15* expression is still not clear. Notably, it is well-known that overexpression of SAT1 would lead to depletion of spermidine and spermine, as well as significant increase of putrescine and N^1-acetyl spermidine [138]. We

speculate that SAT1 may indirectly regulate the expression of *ALOX15* through affecting the cellular polyamine levels. Thus, further investigations are required to explore the role of polyamines on transcriptional regulation of *ALOX15*, as well as the precise role of ALOX15 in modulating ferroptosis.

Taken together, this study revealed a new p53 metabolic target SAT1 that contributes to p53-mediated ROS response and ferroptosis, as well as provided novel insight on the regulation of ferroptosis through polyamine metabolism.

5.3 Differential regulation of p53 transcriptional activity by acetylation

Our identification of two novel p53 metabolic targets ultimately led to the puzzling question of how p53 can differentially regulate a diverse array of target genes in a context-specific manner, and which function contributes to tumor suppression.

Acetylation of p53 is known to play a pivotal role in controlling promoter-specific activation of p53 targets. Previously, we have demonstrated that acetylation at K117/161/162 in mouse p53 (K120/164 in human p53) is required for the transactivation of target genes involved growth arrest, apoptosis, and senescence. In this study, we uncovered another two p53 acetylation sites at lysine K101 and K139, within the human p53 DNA-binding domain, which are acetylated by CBP and Tip60 respectively. Both lysine residues are evolutionarily conserved and are homologous to lysine K98 and K136 in mouse p53. We found that ablation of acetylation at K98 or K136 in addition to K117/161/162 impaired the ability of p53 to activate *TIGAR* and *SAT1*, and repress *SLC7A11*. As expected, simultaneous mutation at all five acetylation sites (5KR: K98/117/136/161/162R) also achieved the same result. These results suggest that K98 and K136 acetylation are both required for the transcriptional regulation of these metabolic target genes. Interestingly, abrogation of acetylation at K98 or K136 alone did not affect transcriptional activity of p53, indicating that acetylation at K98 and K136 may have redundant roles with K117/161/162 in promoter-specific regulation of *TIGAR*, *SAT1*, and *SLC7A11*. Although our

discoveries further support the role of p53 acetylation in promoter-specific transcriptional activation, the molecular mechanism of such differential regulation is largely unknown. Notably, acetylation in the DNA-binding domain does not appear to affect promoter-specific binding, as $p53^{3KR}$, $p53^{4KR98}$, $p53^{4KR136}$, and $p53^{5KR}$ all bind to *TIGAR* and *SLC7A11* gene promoters to the similar extent, regardless of their differential transcriptional activities. One hypothesis is that the acetylation in the DNA-binding domain could modulate the conformation of p53, which in turn influences the recruitment of transcriptional machinery, co-activators, or co-repressors instead of affecting DNA-binding. Histone acetyltransferases (such as Tip60, CBP, p300, and PCAF) are often recruited by transcription factor to acetylate histones and relieve chromatin coiling structure to activate transcription. Therefore, it is possible that different combination of acetylation in the p53 DNA-binding domain allows for the recruitment of various co-activators and thus differentially regulates the transactivation of downstream target genes. Further investigations are required to elucidate the mechanism behind differential regulation by dissecting the co-activators or co-repressors that bind to different acetylation-deficient p53 mutants in a promoter-specific manner.

5.4 The roles of ferroptosis and mTOR regulation in p53-mediated tumor suppression

The discovery of the two novel acetylation sites have broaden the scope of differential regulation by acetylation, and allows us to further evaluate the role of non-canonical functions in p53-mediated tumor suppression by comparing the tumor onset in mice harboring different acetylation-deficient p53 mutants (*p53*$^{3KR/3KR}$, *p53*$^{4KR98/4KR98}$, *p53*$^{4KR136/4KR136}$, and *p53*$^{5KR/5KR}$). As previously described, although 3 of the 27 *p53*$^{3KR/3KR}$ mice developed tumors, sequence analysis revealed that all of those tumor tissues obtained additional mutations in the *p53*^{3KR} gene [97]. This suggests that p53^{3KR} indeed retains intact function as a tumor suppressor. In this study, we found that 26 of the 36 *p53*$^{4KR98/4KR98}$ mice, 6 of the 11 *p53*$^{4KR136/4KR136}$ mice, and all of the *p53*$^{5KR/5KR}$ mice (n=35) developed spontaneous tumors within 18 months of age, mainly including thymoma, angiosarcoma, and sarcoma. Interestingly, unlike *p53*$^{3KR/3KR}$ tumors, sequence analysis showed that there are no additional mutations in the p53 gene in the tumor tissues of *p53*$^{4KR98/4KR98}$, *p53*$^{4KR136/4KR136}$, and *p53*$^{5KR/5KR}$ mice. This clearly indicates that loss of p53 non-canonical functions resulting from additional ablation of K98 or K136 acetylation could significantly disrupt the ability of p53 to suppress tumor formation. Notably, TIGAR, a major p53 metabolic target (also retained by p53^{3KR}) in regulating glycolysis and antioxidant defense, can no longer be induced by p53^{4KR98}, p53^{4KR136}, and p53^{5KR}. However, it is unlikely that this metabolic regulation is a major contributor to p53-mediated tumor suppression, as TIGAR, once

thought to mediate tumor suppression through maintaining cellular free radical homeostasis, was later revealed to promote tumor survival [102]. Moreover, our study also revealed that $p53^{4KR98}$, $p53^{4KR136}$, and $p53^{5KR}$ have lost the ability to transcriptionally activate *SAT1* and repress *SLC7A11*, which are two metabolic targets of p53 (also retained by $p53^{3KR}$) involved in ferroptosis regulation. Additionally, $p53^{4KR98}$, $p53^{4KR136}$, and $p53^{5KR}$ are all defective in inducing ferroptotic cell death upon oxidative stress (Erastin treatment). This implies that p53-mediated ferroptosis may function as the last barrier to defend against tumorigenesis when growth arrest and apoptotic functions are absent in the $p53^{3KR/3KR}$ mice. In fact, some previous studies have also supported the role of ferroptosis in regulating tumorigenesis. First, reconstitution of robust SLC7A11 expression in $p53^{3KR}$ cancer cells significantly abrogated its ability to suppress tumor formation in xenograft mouse models [81]. Second, *Ptgs2*, a molecular marker of ferroptosis, was found to be up-regulated in the tumors when *SAT1* is induced to suppress tumor growth, suggesting that ferroptosis is involved, at least, in part, in tumor suppression. Moreover, *SL7A11* overexpression and *SAT1* down-regulation are observed in several types of human cancers. Nevertheless, it remains to be further elucidated regarding the link between ferroptosis and tumor suppression. It would be interesting to examine if blocking ferroptosis directly by using ferroptosis inhibitor (such as ferrostatin-1) in the $p53^{3KR/3KR}$ mice could disrupt p53-mediate

tumor suppression. We are also generating *p53*$^{5KR/5KR}$*/Slc7a11*$^{-/-}$ mice to see if they will develop delayed tumorigenesis comparing to *p53*$^{5KR/5KR}$*/Slc7a11*$^{+/+}$ mice.

Interestingly, we also found that although most of the *p53*$^{4KR98/4KR98}$ and *p53*$^{4KR136/4KR136}$ mice developed spontaneous tumors within 18 months of age, their tumor onset was significantly delayed for 6 months comparing with *p53*$^{5KR/5KR}$ mice. This suggests that the remaining cellular functions that are retained in p53^{4KR} but further lost in p53^{5KR} are responsible for delaying tumor progression. Notably, although p53^{4KR98} has lost the ability to induce ferroptosis, it still retains the activity to suppress mTOR signaling through activating the expression of two mTOR negative regulators, Sestrin2 and DDIT4 [168, 175]. In contrast, p53^{5KR} has no activity in transcriptionally activation of these two targets, and it does not alter mTOR signaling either. It is well-known that mTOR is a central regulator of cell growth and proliferation through integrating a variety of cellular signaling cascades initiated by growth factors, nutrient or energy stresses, and regulates downstream cellular processes including protein synthesis, autophagy, and ribosomal biogenesis. Thus, appropriate response to these cellular stresses through mTOR can prevent cells from uncontrolled growth and proliferation when nutrient and energy levels are low, and therefore, maintain homeostasis. In fact, inappropriate activation of mTOR due to the deregulation of multiple elements in the mTOR pathway (such as loss of *PTEN*, *PI3K* mutation/amplification, AKT, S6K, and eIF4E overexpression) has been implicated in numerous

types of human cancers, and has been shown to contribute to tumor progression [176]. Since lack of nutrient, oxygen, and energy is a common phenomenon in the tumor microenvironment, it is likely that cancer cells insensitive to these stresses can acquire growth advantage [158]. The link between p53 and mTOR signaling also suggests that mTOR inhibition may be a potential novel mechanism by which p53 suppresses tumor progression. In fact, pharmacological inhibition of mTOR by rapamycin or rapamycin analogues (such as deforolimus, everolimus, and temsirolimus) has been efficacious in several clinical trials for the treatment of a subset of human cancers, and temsirolimus as well as everolimus were recently approved by FDA for the treatment of renal cell carcinoma, giant cell astrocytoma, and PNET (progressive endocrine tumours of pancreatic origin) [176-178]. Efforts in elucidating the role of mTOR inhibition in p53-mediated tumor suppression are still underway. We will be assessing the effect of rapamycin in $p53^{5KR/5KR}$ mice by feeding them with rapamycin-containing food to determine if mTOR inhibition by rapamycin can delay the onset of tumorigenesis mediated $p53^{5KR}$.

In summary, our studies have broadened the scope of non-canonical functions of p53. We first identified a novel p53 metabolic target, PHGDH, which catalyzes the rate-limiting step of serine synthesis. Although it is unknown whether PHGDH participates in p53-mediated tumor suppression, our findings provide novel mechanistic insights into how p53 promote apoptosis in cancer cells through the regulation of the oncogenic serine metabolism pathway. We next characterized another p53 target gene *SAT1* in polyamine metabolism pathway, and elucidated its novel function in ferroptotic cell death responses and tumor suppression. Finally, we have further delved into the question of promoter-specific regulation of gene transcription by p53 acetylation. Our data revealed that two novel p53 acetylation at K101 and K139 (K98 and K136 in mouse p53) is crucial for differential regulation of downstream metabolic targets, and linked p53's function in ferroptosis and mTOR signaling to its role in preventing tumor initiation and progression.

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APPENDIX

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